

How to deal with dreadfulness

Several UN interventions in the past two years betoken an urgent need for more effective ways of deciding when international action is needed. The United Nations should seek help from some judicial process.

THE paralysis with which the world has dealt, or failed to deal, with the succession of crises in the past few years in ex-Yugoslavia, Somalia, Haiti and Rwanda is shaming in the literal sense: it makes people ashamed of their impotence. So, of course, it should. In each place, there has been a civil war in which people have been killed not merely in combat between rival factions struggling for power, but because of their allegiance, supposed or real, to one or the other side. Worse, the killing and deprivation has often been determined by purely cultural considerations — religion (Bosnia), tribal membership (Rwanda) or political opinion (Haiti). The chilling phrase 'ethnic cleansing' has entered the vocabulary. The familiar but equally chilling 'genocide' applies to what has been happening in Rwanda.

Why has there recently been such a spate of dreadful occurrences? There is something (but not much) in the view that they have been made to seem so dreadful only because the television cameras now bring them to everybody's notice. On this view, the world has always been a dreadful place, but most people never learned of dreadful happenings, or did so only so long after the event that their intervention was never on the cards. That is only part of the truth. The widely reported famines in northeast Africa a decade ago were, after all, a direct consequence of the conflict that prompted President George Bush to seek a UN mandate for intervention in Somalia at the end of 1992; government in Haiti has been a scandal for even longer. The degree to which the world's television cameras can be blamed for the current dreadfulness is that they are now less preoccupied with other happenings — the Vietnam War, for example, or the collapse of the Berlin Wall.

A more substantial part of the explanation is that the Cold War has ended. That, with two years delay, was the direct

cause of the tumultuous break-up of Yugoslavia. In a different way, the ending of tension between the two once-superpowers has made Haiti stick out like a sore thumb when previously it could be ignored. And nobody can tell to what extent the murder and enforced emigration from Rwanda is the result of the ease with which modern armaments, surplus to Cold War requirements, can now be bought on the black-market. But when the tools of conflict are so readily to hand, who can be surprised that they will be more widely used?

That, by itself, is a pointer to one step that should be taken. Not so long ago, the then prime minister of Japan urged on the United Nations an international register of arms sales. The chief objective, then, was to hope to understand the consequences of the proliferation of weapons in the Middle East, but the need is more general. Moreover, the ingenuity of private arms dealers notwithstanding, national governments have powerful reasons for ensuring that the weapons they allow to be manufactured in their territory should not find their way into the hands of potential enemies. The United Nations (UN) should take up Mr Toshiki Kaifu's plan as a matter of urgency.

But the crux of the problem of recurring dreadfulness is different. The United Nations charter, which has the status of a treaty between its members, does not provide for interference by one member or group of members in the internal affairs of another state. Without that understanding, no treaty would have been signed in San Francisco in 1945. The Soviet Union is as then only the most conspicuous of the states that would have objected. And even now, external intervention is against the rules. The possible intervention in Haiti is based only on the proposition that the legitimate government has not been allowed to continue in office by the local warlords. The same is true of the French-led intervention in Rwanda last month; that was legitimized by the UN Security Council on humanitarian grounds alone. That is plainly insufficient to meet the wishes of the television-watching world that the outrageous murder of some section of the population should be halted. The problem for the international community is to distinguish between acts of that kind on that scale and equally barbarous behaviour towards individuals, of which there is also too much.

The most obvious need is for some judicial process that will determine when a regime has overstepped the bounds of decency towards those under its control. The International Court of Justice is too slow. The ideal would be that the UN should appoint a judicial commission on the request of a

One-day conferences

A *Nature* meeting, *Genetics in Europe now*, will be held in Berlin on Friday 30 September 1994 at the Brandenburg Akademie, and the cost is DM195 or £75 (plus VAT).

A meeting in Paris, *What is distinctive about European science?*, will be held on Wednesday 26 October 1994 at the Ecole Normale Supérieure. The cost is FF675 or £75 (plus VAT).

Payment may be made by cheque (payable to Macmillan Subscriptions Ltd) or credit card. For further details of either meeting, or to reserve places, please write, telephone or fax to Pippa Burnett, Nature Conferences Office, 4 Little Essex Street, London WC2R 3LF, UK; telephone +44 71 836 6633 ext. 2593; fax +44 71 379 5417.



significant number of its members, and that the commission's report should be made public in advance of a decision by the Security Council. To some degree, the sovereignty of member-states would be undermined by such devices, but as the world knows by now, the days of untrammelled sovereignty are coming to an end. □

Replication defined

Those who test others' ideas by repeating their experiments are not required to do so slavishly.

THE letters from Dr Jacques Benveniste and others and from Fred A. C. Wiegant appearing (much delayed) on page 322 have been stimulated by the publication last year (Hirst *et al.* *Nature* **366**, 525–527; 1993) of an account of a test of Benveniste *et al.*'s earlier report of the biological reactivity of even indefinitely diluted solutions of anti-IgE. They raise an interesting point about the role of replication in science — one on which confusion can arise, or can be made to arise. Hirst *et al.* set out to test Benveniste *et al.*'s conclusions by repeating his measurements, but Benveniste now says that, because the repetition was not exact, their negative result does not prove his conclusion wrong. Technically, of course, he is correct. It is, indeed, possible that one of the variations from Benveniste's procedure in that followed by Hirst *et al.* — Benveniste claims to have identified fifteen — accounts for the difference between the two groups's conclusions. Then, so what?

Dilemmas of this kind are not as infrequent as may be thought. It is not so long ago (just under a decade) since J. J. Simpson published data suggesting that there is a neutrino with a mass of 17 keV, for example. The implications were startling for cosmologists. But the notion is now dead, because exact replications of the original measurements and independent tests of the same conclusion, often more sensitive, failed to confirm it (see *Nature* **366**, 29–32; 1993). The same has happened with the hunt for the so-called 'fifth force', first suggested (by experiment) as a force of intermediate range between all massive objects (see *Nature* **356**, 207–32; 1993). The sheer variety of the measurements by which confirmation was sought lent conviction to the conclusion that there is no fifth force, but has nonetheless left gravimetry much richer.

It is unfortunate, and a little sad, that Benveniste and his colleagues do not appreciate the parallel. Correctly, Hirst *et al.* did not conclude in their article that Benveniste was mistaken, but merely that their reasonable test of his conclusion failed to support it. It is for readers of this arcane branch of the literature to make up their own minds what weight to give to the handful of published investigations of the memory of water. For Benveniste, apparently still convinced that water does have a memory, the proper course would be to regard the differences between his procedure and that of Hirst *et al.* as pointers to the attributes of his own procedure that may be responsible for the results on which he has been brooding these past six years. □

Waldegrave's departure

The transfer to another ministry of Britain's first science minister for three decades is a loss.

THE history of science ministers in Britain is sparse, to say the best of it. There was a brief spell in 1962–63 when Lord Hailsham, a combative Conservative party warhorse, held the post on a part-time basis until it disappeared beneath the hoopla of the arrival of a Labour government in 1964. A brand-new Ministry of Technology was created to give bite to the government's policy of regeneration through "white-hot technology", science was bundled in with education, and the lack of a minister with even part-time responsibility in the field became apparent only gradually. Then, two years ago, the present British government recreated the post, at least in part because of the urging of the House of Lords that something should be done.

British science has been lucky in the first incumbent of the new post, Mr William Waldegrave. Whatever disagreements there may have been and may persist, his spell at the newly created Office of Science and Technology has been intellectually distinguished and robust. He has listened to what people in the research community have been saying, he has engaged in argument about controversial policies and he has been vigorous in putting in place the organization his policies have made necessary. Disagreements about them apart, it can only have been good for science in Britain to have been dealt with intelligently, for just under two years, and made to seem important. Indeed, the radical reorganization of the research councils he has carried through might not have been possible if he had been personally less engaged.

But now, as is the habit of British governments, Waldegrave has been snatched away to become the minister of agriculture (see *Nature* **370**, 237; 1994). His new brief may include a few scraps of science, bovine spongiform encephalitis (BSE) among other things, but the demanding parts of it will have to do with the European Union's own protection racket, known as the Common Agricultural Policy, which the British government wishes to dismantle (or 'reform'), against the wishes of most of its partners. It is a great pity that one with a proven flair for making links with the research community should have to spend the next few years of his political life arguing the toss with farming lobbies on such arcane matters as the value of the 'green pound' (which is the accounting unit used in converting British agricultural prices into European units).

Waldegrave's successor, Mr David Hunt, is also personable and intelligent. The worry is that his brief (within the British Cabinet Office) includes an ill-defined responsibility for the government's political success. It is as if he is meant to play for Mr John Major, the prime minister, the role Mr James Baker took on for President George Bush. It is unlikely that science will be able to command as much of his attention as it did of Waldegrave's. That will be especially unfortunate when the inevitable teething troubles (and perhaps worse) of a new organization will have to be endured. □

Rockefeller denies work pressures led to 'poisoning' of researchers

New York. Rockefeller University in New York, one of the world's leading research universities, has been forced to defend itself against charges that excessive work pressure on postdoctoral students led to a bizarre incident in which one student is thought to have 'poisoned' the coffee of a dozen academic colleagues.

The incident took place in June in the laboratory of Robert Roeder, a molecular biologist whose research group is investigating the mechanisms of transcription. It was followed the next evening by the discovery that gas to all the laboratory's Bunsen burners had been switched on, but left unlit — and subsequently by a small fire in a stockroom and letters containing death threats being received by two female researchers.

Roeder himself also received threatening letters. The university called in the police to investigate and also increased security; all those entering the laboratory, for example, must now pass through a metal detector. But it had been hoping that the matter could be resolved internally.

Last week, however, the story was leaked to the *Wall Street Journal*. The normally

staid financial newspaper carried a front-page story under the attention-grabbing headline "Who's trying to kill the great biologists of Rockefeller U.?"

The story was quickly picked up by local papers in New York, many of which have since been providing daily embellishments based on information from police sources. (One prominent columnist described the "mad menace of Rockefeller University" as "the perfect summer story in Sin City".)

The original article detailed how several coffee-drinking researchers in the laboratory simultaneously experienced symptoms of diarrhoea and vomiting. The subsequent letters to the women researchers claimed this had been caused by sodium fluoride, known to be lethal at high dosages.

In describing this and the other incidents, the *Wall Street Journal* quoted a university official as suggesting that a possible contributory factor was tension among laboratory researchers caused by the continuous pressure to produce top-quality research.

Since then, however, other officials have moved to counter any impression that research staff in the laboratory — or indeed elsewhere at Rockefeller, which has in re-

cent years been carrying out a wide reappraisal of the way it organizes research and of the powers of laboratory heads — are required to work any harder than at other leading research universities.

"I think it is unfair that Roeder should be pointed out as some villain in this," says Torsten Wiesel, the 1981 Nobel prizewinner in medicine, who took over as president of Rockefeller two years ago following the resignation of the molecular biologist David Baltimore.

Wiesel says there is "a certain intensity to one's activity" in all laboratories that are "serious" about science. "We are all under pressure to produce interesting results," he says. "This is part of the reality of life."

The researchers in the laboratory have also jumped to Roeder's defence. A letter to *Nature* signed by almost 40 postdoctoral researchers, postgraduate students and other staff (see panel) acknowledges that pressures exist in Roeder's department. But it claims that most of them "are derived from our own drive and ambition".

Sean Stevens, the main organizer of the letter, says that the signatories are keen to show that "it is not us, it is not the research we do, and it is not the laboratory" that have been responsible for the recent events. He challenges statements in the press from an unnamed former researcher that Roeder's abrasive manner had led to "huge fights in [his] laboratory".

Since the original newspaper articles appeared, attention has shifted from work pressure to the more familiar territory of personal relationships. Last Thursday (28 July), the police confirmed that, largely on the basis of a psychological profile of the perpetrator, their main suspect was a foreign postgraduate researcher whose approaches had apparently been spurned by one of the women who received a death threat — and who felt that he was being ignored by the other.

Although a DNA test failed to match a saliva sample taken from one of the letters containing the threat to the suspect, police authorities said on Monday that they remain confident in their identification, and that "the investigation is continuing" in the search for firm confirmation.

Wiesel says he is confident that the incident will not harm the reputation of the university. "This might have happened anywhere," says Wiesel. "Next time, it might be in Cambridge, England." But with no firm confirmation of the perpetrator — and at least one researcher claiming she is now scared to come into the laboratory — tensions remain high.

David Dickson

'Drive is from within', say scientists

The following letter has been received by *Nature* from research, administrative and technical staff in Robert Roeder's laboratory at Rockefeller University:

Sir—Many of your readers are aware of a series of disruptive incidents that have occurred within the laboratory of Dr Robert Roeder at the Rockefeller University. As members of Roeder's group, we felt it necessary to address, especially to the scientific community, the provocative and unfair statements made in the media about our laboratory.

Our laboratory has done, and continues to do, outstanding work in the area of transcription. Like any major laboratory competing in a 'hot topic' there are pressures, most of them arising from our own drive and ambition. The competitive environment in which we work in no way justifies the destructive acts perpetrated against our laboratory. Most of us conduct our research in an atmosphere of considerable collegiality, cooperation and friendship. While it is true, as in any large group of people, that there are occasional disagreements, reports of huge fights or racial tension are completely false.

The success of our laboratory can in large part be attributed to our mentor's determination and support. While Roeder is demanding, he is no harder on those who work with him than he is upon himself, and he has been very generous in providing an excellent environment in which

to carry out research. We believe that the past and present record of scientific productivity of our laboratory speaks most eloquently for the work environment.

Any suggestion that the incidents that have occurred are somehow justified by the laboratory environment is ridiculous. A profoundly disturbed individual has chosen illegal and destructive means to solve his or her personal problems, disrupting the lives and work of the people in our laboratory. We hope that we can be judged on the basis of our record of scientific achievements and not unattributed aspersions cast in the popular press.

SEAN STEVENS, HUI GE, THOMAS OELGESCHLAGER, FRANCK BRUNEL, XIAOLU SHI, TAE KOOK KIM, YURIKO SUZUKI, LUZ HERMIDA, JOSEPH FONDELL, SHWU-YUAN WU, MARCUS KRETZSCHMAR, ALEXANDER HOFFMANN, ZHENGXIN WANG, SUSUMU INAMOTO, YOSHIKI OHKUMA, CAMILO PARADA, ERNEST MARTINEZ, CHENG-MING CHIANG, YING-JU HSIEH, MAGDA CARVALHO, DONG KUN LEE, YAN LUO, JONG-BOK YOON, TSUTOMU OHTA, UNKYU KIM, MARIA POPOV, RICHARD BERNSTEIN, AMITABHA CHAKRABARTI, BETH MOORFIELD, LEANNE GONZALEZ, JIAN-QING HUANG, CARMEN-GLORIA BALMACEIDA, JEFF DEJONG, LIN BAI, XIAOHONG ZHANG, MOHAMED GUERMAH

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'Lack of behaviour studies is hampering AIDS prevention'

Washington. The United States should fund a national survey to fill gaps in current knowledge of sexual practices and drug use and their links to HIV infection, according to a panel of experts convened by the Institute of Medicine (IOM). In a report published last week, the panel says that the lack of such knowledge is hampering efforts to prevent the spread of AIDS.

In the past, requests for federal funding for any survey that includes questions about sexual practices have been opposed by powerful conservative voices in Congress, backed by fierce grass-roots opposition through the television evangelist network.

The IOM panel's clear call for a federally funded survey of sex and drug use means that members of Congress will once again have to face the issue, and decide whether to give in to pressure from the religious right.

The IOM report was commissioned by Congress in 1992 in an attempt to assess the balance of biomedical and behavioural research conducted in the National Institute of Alcohol Abuse and Alcoholism, the National Institute of Drug Abuse and the National Institute of Mental Health.

It was prompted by AIDS activists who felt that not enough was being spent on research in the behavioural and social sciences to help public health officials devise more effective prevention techniques.

According to one congressional aide, now that the IOM has endorsed a full survey, the need is for scientists at the National Institutes of Health to add their backing. The Bush administration had refused to provide support. "But the political climate has changed, and there is more chance that a sex survey would get federal funding, although it is not certain."

Mindy Thompson-Fullilove, associate professor of clinical psychology and public health at Columbia University in New York, and a member of the IOM panel, points out that the United States carries out annual surveys of drug use and violence, which are illegal, but not about legal sexual practices. "Without the information from such a survey, we all feel profoundly crippled in attempts to devise effective prevention messages," she says.

Fullilove claims that the anti-AIDS messages put out by the Public Health Service have not been sufficiently frequent, frank or well-targeted. "We need to know a lot more, say, about how people view barrier protection," she says. "A lot of people say they don't like condoms, but some research suggests that they might have used them once and had a bad experience. If that is the case then the advertisement would be different than for people who have never used them."

Sex surveys have been conducted in the past in the United States, but with private funds. One, at the University of Chicago, started out by requesting federal funds, but the political opposition was so strong that a private foundation took over.

Another survey, conducted by the Center for AIDS Prevention Studies in San Francisco, canvassed 14,000 people by telephone. But critics of such an approach say that many of those who should be reached by a survey may not have a telephone. "We need a national survey for the rational development of public policy," says Fullilove.

The report also calls for more research on the social behaviour of individuals. "We know very little about what pressures are brought to bear on a person by peers or families or communities," says Judy Auerbach, the panel's study director. Fullilove, for example, points out that it is often unacceptable for Hispanic women to talk about sex, and that this may affect their ability to negotiate the use of a condom.

Another example of potentially useful social science research, says Fullilove, is detailed mapping by medical geographers showing the location of infected individuals on a street-by-street basis. The first use of medical mapping was in London in 1854, when an outbreak of cholera was traced to a single water pump. "We already know that HIV infection correlates strongly with urban decay, [but] we need to know what is analogous to the water pump," says Fullilove.

Finally the panel says that a better understanding is needed of how the AIDS epidemic interacts with, for example, tuberculosis and violence. At times the immune system 'locks up' HIV, but at others — such as when the patient has tuberculosis — it does not. "Does the TB epidemic affect the HIV epidemic?" asks Fullilove. "We don't know, and we need to know." **Helen Gavaghan**

UK insurers drop enquiry on HIV testing

London. The Association of British Life Insurers (ABI) last week advised its member insurance companies to stop asking applicants whether they have had HIV/AIDS tests or counselling. But the association is still recommending that applicants be asked if they have ever received either a positive test result, or treatment for HIV/AIDS or hepatitis.

The move reflects the fact that the disease has had a smaller impact on insurance payouts than originally predicted. Since 1987, when the question was introduced on application forms, British insurers have paid out more than £50 million for deaths in which HIV or AIDS

Minister is rebuffed over bid to require publishing in French

Paris. French research organizations breathed a sigh of relief last week when the country's constitutional council rejected a provision of a new law that would have required virtually all publicly funded research to be published in French. The law would have allowed the research ministry to make individual exemptions. But the constitutional council rejected the whole provision as contrary to "freedom of communication and expression in teaching and research".

The provision was contained in a wide-ranging law on the use of the French language drawn up by Jacques Toubon, the minister of culture, and recently adopted by both the National Assembly and Senate.

The council's decision was part of a longer judgement rejecting various elements of the new law. A long campaign by research organizations — in particular, the French Academy of Sciences — to have other restrictive provisions watered down also seems to have been successful.

For example, although the council has approved an article giving all participants at scientific meetings in France the "right to express themselves in French", the article no longer insists on simultaneous translation.

The Centre National de la Recherche Scientifique (CNRS), the country's largest research body, bluntly told the government that this would cost it FF10 million (US\$1.84 million) a year.

The council has approved the requirement that conference proceedings from such events should carry at least an abstract in French. Such abstracts will also be required in foreign journals or magazines that receive subsidies from French public bodies.

But research organizations are confident that the decree, yet to be published, in which the government specifies how these and other provisions are to be enforced, will take account of their concerns. **Declan Butler**

was implicated, considerably less than had been anticipated.

Insurance companies had previously wanted to be able either to refuse a policy or to charge higher premiums to an applicant who had taken a test, even if the test results were negative. This was based on the assumption that only people who consider themselves at high risk were likely to have taken a test.

But the ABI, in recommending a change which has been widely welcomed by AIDS charities, admits that testing for HIV/AIDS is now more widespread than anticipated, and is used by individuals for many reasons. **Maggie Verrall**

US wants to lift R&D spending to 3% of GDP

Washington. The United States should raise its total spending on research and development (R&D) from 2.6 per cent of gross domestic product to around 3 per cent, according to a long-awaited science 'white paper' due to be released this week by the Clinton administration.

The policy document is the first such statement by a US administration since 1979. It proposes that this "modest increment" should be shared by the federal government and the private sector. But it does not give a date by which it suggests that this target should be met.

The white paper, entitled *Science in the National Interest*, was due to be released yesterday by vice president Al Gore. It sets five goals for US science: world leadership "across the frontiers of scientific knowledge"; better connections between research and national goals; more partnership with industry; a better education for scientists and engineers; and improved scientific education for the public at large.

The policy document — which had been criticized before its release for failing to address specifics (see *Nature* 370, 5; 1994) — also says that technology and fundamental science depend on each other, and should not be regarded as competitors for funds. "Today's science and technology enterprise

is more like an ecosystem than a production line," it says.

This updates the view of Vannevar Bush, who laid down the framework for US government support for science 50 years ago in his report *Science: The Endless Frontier*. The new document says that Bush's conception of "a competition between basic and applied research" has been superseded by an "interdependence of basic research, applied research and technology".

The document lists a number of critical problems facing US science, including a lack of cash for new university laboratories, a lack of mission for government laboratories, and the need for new mechanisms to fund large international or long-term projects. But rather than offering solutions, it tends to refer such problems back to Clinton's cabinet-level National Science and Technology Council (NSTC).

M. R. C. Greenwood of the White House's Office of Science and Technology Policy (OSTP), who co-ordinated the preparation of the paper, says however that its main objective was to clarify the reasons for investing in fundamental research. "It makes a clear statement that this is a critical investment that must be made, even in a tight fiscal environment," she says.

The document pledges a cross-govern-

ment review of all federal laboratories, and repeats proposals for tax changes to encourage industrial R&D and private-sector contributions to new university research facilities. Noting the need for billions of dollars to renovate university laboratories, it promises that the NSTC will "develop options for how to implement the federal investment as a systematic, long-term, multi-agency, merit-reviewed program".

The NSTC will also, it pledges, "recommend policies for long-term multinational agreements for the support of large scientific projects". The administration also says it will "work with Congress to find mechanisms for long-term authorization and budgeting commitments" for large US and international projects.

The administration has also named the 19 members of the President's Committee of Advisors on Science and Technology (PCAST), the body announced last November to support the NSTC. It will be co-chaired by OSTP director John Gibbons and former Hewlett-Packard boss John Young. Its members include Peter Raven, director of the Missouri Botanical Garden, Charles Vest, president of the Massachusetts Institute of Technology, other senior academics and half-a-dozen industrial chief executives.

Colin Macilwain

Europe weighs bid to extend life of sensing satellite

Paris. A proposal from the European Space Agency (ESA) to extend the operation of its remote-sensing satellite ERS-1 beyond the end of the year, enabling it to operate in tandem with its successor ERS-2, is hanging in the balance. The 15 member states of ESA involved in the programme say that they may have difficulty in finding the small amount of further funding needed.

ESA had hoped to keep ERS-1 operating for a further year, after almost half of the 400 proposals for ERS-2 experiments asked for ERS-1 to be kept in operation in tandem (40 proposals depend on tandem operation). Scientists say that if ERS-1 is shut down in December, an opportunity to carry out unique research at little cost, using the two satellites simultaneously, will have been wasted.

The ECU920 million (US\$1.3 billion) ERS-1 was launched in 1991. Its main instruments are a radar altimeter, which gives precise measurements of the height of the ocean surface and ice sheets, and a synthetic aperture radar, which provides images with a resolution of 10 cm that can also be used for interferometry.

ERS-1 is regarded as one of ESA's most successful science missions, and the agency has had no difficulty finding backers for ERS-2. But even last year it had to struggle to raise the relatively small sum needed to

operate the satellite for a third year. Member states have so far failed to agree whether to provide the ECU8 million it would cost to keep ERS-1 running through 1995.

ERS-2 is scheduled for launch around Christmas, although it will not enter service

"best ice thickness maps of the Antarctic we've ever had". He says that ERS-1's synthetic aperture radar — which penetrates fogs and snowstorms — has been providing high quality pictures of the ice surface and of fractures and crevassing within it.

Interferometry, comparing two pictures of the same area taken at different times, when combined with radar altimeter data, is giving "a completely new understanding of the dynamics of ice sheets", says Vaughan.

But ERS-1 revisits the same location every three, 35 or 186 days, depending on which orbiting cycle it is in, while 35 days is the maximum time limit for interferometry. Using the satellite in tandem with ERS-2 would reduce the time between visits to one or eight days, and also increase the area covered. "With ERS-1 we can do some interferometry, but flying ERS-1 and 2 together we could do the whole [Antarctic] continent," says Vaughan.

An official at the British National Space Centre says that money is likely to be forthcoming to keep ERS-1 in operation during the three months before ERS-2 becomes operational. But he adds that scientists will have to make a full case for further tandem operations if this is to win funding at the next meeting of the board in September.

Declan Butler

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

ERS-1: failure to prolong mission 'would waste an invaluable opportunity'.

until three months later. ESA wants to keep ERS-1 in operation during this three months, and then run it in tandem with ERS-2 for a further nine months.

One ERS-1 user, David Vaughan of the British Antarctic Survey, says that ERS-1's radar altimeter has already provided the

David Parker/ESA/SPL

Science council returns to political favour

Tokyo. One of Japan's leading brain researchers, Masao Ito, was elected last week as the new chairman of the Science Council of Japan, the government's main academic advisory body on science policy. Ito succeeds Jiro Kondo, who has headed the council for the past nine years. His appointment comes at a time of growing influence of the council on government policy.

The council has 210 members elected every three years by nearly a thousand registered scientific societies, including those in social science, engineering and medicine, and receives a small budget from the prime minister's office.

The council fell out of favour with the government in the 1970s and 1980s largely because it was seen as a "nest of communists" that always disagreed with government policy — it had opposed all forms of nuclear power, and repeatedly disagreed with the government on education policy.

At that time, all Japanese scientists had the right to elect members of the council. But voting fell prey to groups of socialists

and communists who, according to one council member, established a "systematic machine" to elect members with left-wing views. As a result, many senior academics shunned membership of the council.



Ito: plans a more active role.

Voting procedures were changed in 1985 to give voting rights to scientific societies rather than individuals. Furthermore, even though only a formality, the prime minister now has to approve the appointment of all elected members.

Kondo took over leadership of the council in 1985 under the new regime, and has served three terms of three years each, during which time the council has returned to government favour, and now includes many of Japan's leading scientists.

Between 1989 and 1993, for example,

when Japan was faced with requests from the United States government to fund the Superconducting Super Collider, the prime minister and Japanese government repeatedly turned to the council for advice.

The government's new appreciation of the council was demonstrated last week when the prime minister, Tomiichi Murayama, invited all 210 members of the council to his residence for the first time.

Ito is a former dean of the medical faculty of Tokyo University and director-general of the Frontier Research Programme at the Institute of Physical and Chemical Research (RIKEN). He is well known internationally for his research on the brain, in particular on synaptic plasticity and the cerebellum, and as one of the architects of the International Human Frontier Science Programme, a foundation that supports research on the brain and biological functions.

Although the reputation and influence of the council has been re-established under Kondo's leadership, it still suffers from what some members call "Japan Science Council disease". This is the tendency for the council to send out questionnaires to the hundreds of thousands of scientists it represents on a particular topic, analyse the results, produce a report — and then forget it.

Ito has already indicated that he intends to give the council an even more active role in setting Japan's science policy than in the recent past. He is quoted in Monday's edition of Japan's leading financial newspaper, the *Nikkei Shimbun*, as saying "the council should not just carry out [the] routine work of the little brain (cerebellum), rather it has to do [the] creative work of the brain proper (cerebrum)".

David Swinbanks

Russia takes steps to block brain-drain

Moscow. The Russian government has adopted a new programme aimed at reducing the emigration of scientists to the West by paying them more to stay at home. Its approach is based primarily on broadening the system of hiring scientists on a contract, rather than a salaried, basis.

The government's decision followed a cabinet discussion in which Boris Saltykov, the minister for science, said that in 1992 alone, 4,500 scientists had left Russia. Overall, he said, the science sector had lost one quarter of its employees in 1993.

The main destinations of scientists leaving the country were the United States, France and Germany. The government has agreed to allocate an additional 42 billion roubles (US\$20 million) to attempt to stem the brain-drain and encourage scientists to remain in Russia. □

Japan backs brain and AIDS research

Tokyo. Research on the brain and on AIDS will receive the biggest single multi-year grants — each worth more than ¥1 billion (US\$10 million) — in next year's 'priority' grants for large teams of researchers announced last week by Japan's Ministry of Education, Science and Culture.

In contrast, this year's 'special distinguished' grants, the ministry's largest grants for individuals which were also announced last week, are concentrated more on the physical sciences, in particular materials science and chemistry. Both type of awards indicate the areas where some of Japan's best basic research is being carried out.

The 23 multi-year priority grants begin next fiscal year which starts on 1 April, giving time for teams of researchers to be assembled. Yoshiyuki Nagai of Tokyo University's Institute of Medical Science will head one such team, which will investigate the pathogenesis and control of AIDS, and will be carried out at about 30 institutions throughout the country.

The team is expected to receive about ¥1.2 billion over three years, but this may be cut back slightly by the Ministry of Finance. This marks a significant boost in the government's spending on AIDS research, at present only a few billion yen a year.

Two even larger grants go to brain research. Takao Shimizu of Tokyo University's faculty of medicine will head a team of about 50 researchers at a dozen institutions investigating molecular mechanisms of synaptic plasticity with a four-year grant expected to exceed ¥1.4 billion. The other

is to Tadaharu Tsumoto of Osaka University's medical school, who will head a group spread among about 20 institutions that hopes to elucidate the functional development of neural circuits at the molecular level.

This follows a general trend by the government to increase support for brain research (see *Nature* **370**, 243; 1994).

Research on advanced computer systems, including virtual reality and 'intelligent' silicon electronic systems, also gets significant support in the priority grants.

In contrast to the priority grants, the special distinguished grants, which provide a few million dollars to individuals for projects lasting between three and five years, take effect immediately. In the 1980s and early 1990s, these grants were dominated by awards in biology. In the past few years, however, the emphasis has shifted to the physical sciences, which account for six of the 10 grants announced last week.

Ministry of Education officials say the shift is because applications from physical sciences have doubled in the past three years, whereas those from biological sciences have remained more or less constant. This reflects the strength of Japan in newly emerging areas of material science, such as high-temperature superconductors.

Outstanding among them is an award of ¥281 million over four years to Akihisa Inoue of Tohoku University's Institute for Materials Research for research aimed at the discovery and development of new types of glass made out of metallic materials with glass-like properties.

D. S.

Judiciary criticized over French blood trial

Paris. The Paris bar association last week warned the French legal system against allowing public opinion to take precedence over the law in its prosecution of individuals accused of supplying haemophiliacs with blood-clotting factors contaminated with HIV in the mid-1980s.

The organization, which represents more than 11,000 barristers, gave its warning after a judge had indicted Michel Garretta, former director-general of the French National Blood Transfusion Service (CNTS), on the criminal charge of 'poisoning'.

"In the absence of any new element, someone already found guilty cannot be retried for the same acts," the association said. Garretta had already been sentenced to four years in prison for the same acts in 1992, but only in a magistrates court, and on the misdemeanour of 'deception over product quality'.

In a communiqué, the association said that "whatever the respect and compassion which the pain of the victims imposes on everyone, we have the right to expect that the judiciary does not cede to the pressure of public opinion". It added: "Only the law should guide its action."

The current legal confusion stems from a ruling by the Supreme Court of Appeals in June. This upheld Garretta's first conviction, rejecting demands by groups representing haemophiliacs that it be overturned and that new charges of poisoning be brought. But the court also left the door open to the further charges by stating that the acts committed could qualify as poisoning.

The bar association's stand also raises the question of how far public pressure has influenced the judiciary throughout the affair. The association says it would be "inappropriate" to comment on the first trial. But at least one senior official says that pressure from the public and the press have prevented justice from being done.

One aspect of concern is the large number of individuals who shared responsibility in the affair, from government officials to physicians who prescribed products they knew were unsafe. The prosecutor at the first trial remarked that "if there are only four charged, it is that the choice was not between four or five, but between four and 100".

It is clear that CNTS — and Garretta in particular — continued to produce clotting factors contaminated with HIV to avoid importing heat-inactivated products. But there is a widespread impression in France that the four convicted officials are in some sense scapegoats.

Indeed, the lawyer representing Garretta, François Morette, said last week that he

intends to bring proceedings against 300 other individuals for their part in distributing contaminated blood products.

Others continue to support Jean-Pierre Allain, the former head of research and development at CNTS. Many scientists feel that his sentence to four years in prison (with two suspended) on the same charges as Garretta was particularly unjust, given that he had opposed the CNTS's policy of not importing heat-inactivated products.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Garretta: concern grows over 're-writing history'.

Jean-Baptiste Brunet, the director of the European Centre for the Epidemiological Monitoring of AIDS, has claimed that the press, the government and the public have all turned a blind eye to many questions left unanswered by the blood trial — which concerned only contamination of clotting factors — and in particular to those about the thousands of patients who were contaminated through whole-blood transfusions (see *Nature* 367, 304; 1994).

One reason for the relative neglect of the latter, according to Brunet and others, is

that, in contrast to the haemophiliacs, most of the individuals concerned settled for compensation of several million francs rather than pressing for criminal proceedings.

A wider concern expressed by some scientists who have worked on AIDS since the start of the epidemic is that history is being rewritten in order to present the contaminated blood affair as a crime perpetrated by a few individuals.

They argue that such a version may be easier to live with than the idea that the affair may have resulted from a deep malfunctioning of French society and institutions that encouraged nationalist and protectionist attitudes, and led to economics being given priority over public health.

Françoise Barré-Sinoussi, for example, a co-discoverer of HIV, has publicly stated that the version of events in 1984 and 1985 as now being described in the media "bore no relation" to the reality facing scientists at the time.

Newspaper articles from 1985 suggest retrospectively that there were enough warning signs in the public domain that blood supplies were contaminated with HIV for the press, the scientific and medical community and haemophiliac groups to have intervened. But, apart from a few individuals, most people failed to recognize the consequences.

Libération, for example, referred in its issue of 8 March 1985 to a study showing that almost one per cent of blood donors in Paris were seropositive, and noted four months later that heat-inactivated blood products were being reserved for seronegative haemophiliacs. But little was made of the issue at the time.

Declan Butler

UK 'should lift superhighway rules'

London. A House of Commons committee has recommended that the British government should lift a ban on British Telecom (BT) and other telephone companies that prevents them from providing entertainment services on fibre-optic cables.

In a report published last week, the Select Committee on Trade and Industry says that uncertainty over whether the government would lift the ban is holding back investment in a new network designed to act as Britain's 'information superhighway'.

The committee also urged the government to rectify what it described as "the lack of a clear sense of vision or excitement" of the potential for broadband communications in the United Kingdom, and praised the high profile given to developments in the United States and elsewhere.

It has been calculated that the construction of the national fibre-optic network will cost £15 billion (US\$23 billion) over and

above investment already planned by the telecommunications industry.

The committee says that this will be recouped by the boost to the national economy. But BT, which is the largest communications company in Britain, has said that it cannot justify such large expenditure without a guarantee that it can use the network to operate lucrative services such as home shopping, videos on demand, home financial services and interactive video games.

In 1984, the British government introduced a ban restricting national public telecommunications operators from transmitting entertainment services. This was intended to stimulate investment in the cable-television market. The ban is not due to be lifted until 2001 at the earliest. But the committee wants the government to make a clear commitment to lift the ban by the end of 2002.

Maggie Verrall

University censured over research accounting

Quebec. Quebec's main research funding agency has joined its federal counterpart in suspending grants to Concordia University and demanding the return of money which has been 'improperly' used, following an audit of the university's engineering and computer science faculty.

The federal body, the Natural Sciences and Engineering Research Council (NSERC), has asked the Royal Canadian Mounted Police to investigate the irregularities. Both reinforce claims by an engineering professor convicted of shooting four academic colleagues that work pressures and malprac-

tice in his department contributed to his actions (see *Nature* 370, 166; 1994).

The Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) said last week that before it will lift the suspension the university must show that it has introduced suitable management measures. The agency provides Concordia with grants worth more than C\$2 million a year.

The FCAR is an autonomous agency of the provincial education department, and was set up in 1984 to promote the scientific and technological development of Quebec. Its grants total about C\$50 million a year.

While NSERC grants are awarded to individual researchers, FCAR grants are the responsibility of the organization to which the applicant is affiliated.

The audit report found that research grant funds "were diverted to uses other than those permitted under the rules, and that costs associated with specific contracts were not properly matched against the revenue from those contracts — and vice-versa".

It said that a combination of university procedures and a "thoroughly confusing computerized accounting system" made it impossible for either the university treasury or its office of research services to ensure that grantors' rules were being adequately followed.

The report claimed that many researchers and teachers in the engineering and computer science faculty "participated in a deliberate scheme to manipulate account balances, transfer amounts of money, co-mingle research grants with contract funds and divert them to discretionary research accounts". All this had been done "in the interest of promoting research", and defended on the basis that the funds were "intended to be used in research anyway".

In some cases, grants and contracts had been charged for personnel and facilities whose cost was already absorbed by the university's operating budget. The resulting revenue went either into accounts held under the discretionary control of the dean, or to other department heads.

Conflicts of interest were also discovered by the FCAR report. In one case, a professor had calculated that he could spend up to 190 days a year working as a consultant to outside bodies — 15 more than he worked for the university.

The report said a "climate of pressure" existed at Concordia. This included pressure from the administration to maximize grants and contracts, which influenced faculty members' evaluation for appointments, tenure and promotion; pressure from peers to break new ground in research; and pressure from granting agencies to compete for funds. This pressure, it said, was "so intense that it overwhelms and blurs the vision of even those not directly benefiting from the improprieties".

David Spurgeon

Correction: European Patent Office

In the article "A moral question for patent legislation" (*Nature* 369, 589; 1994), part of the explanation of the "Inventive step" (column 1) was ambiguous. The second sentence of this section should have read: "The EPO will want to be satisfied that ... the invention involves the exercise of ability beyond that to be expected of the person 'skilled in the art'."

Second, the caption to the chart under the heading "Recession hits European patents" (*Nature* 369, 694; 1994), should have referred to the distribution of patents applied for — not awarded — at the EPO.

India bans the use of sex screening tests

New Delhi. The Indian parliament last week passed a bill banning the use of prenatal tests to detect the sex of unborn children. Doctors who perform the so-called 'sex determining tests' (SDTs) for selective abortion of female fetuses — as well as women who demand them — face up to three years in jail and Rs10,000 (US\$323) fine.

The move is intended to check a rapid increase in the killing of female fetuses in a country where girls are still considered a financial burden because of the dowry that has to be paid when they marry. This can range from a few thousand rupees in middle-class families to several hundred thousand rupees in the most affluent social groups.

India's liberal abortion laws, as well as the general availability of SDTs since the late 1970s, have together provided a way out of the dowry problem. For as little as Rs500, the private clinics that have sprung up all over the country offer a service using ultrasound, amniocentesis and chorionic villus sampling techniques. Official figures are not available, but voluntary organizations claim that between 50,000 and 80,000 female fetuses are aborted every year after the sex test.

According to the 1991 census, the sex ratio has fallen to 929 women for every 1,000 men, compared to 972 women in 1901. Selective abortions, increasing every year, might distort the sex ratio even more.

The legislation is intended to curb this threat by halting the rapid growth of sex clinics, and making women's access to them difficult. In future, sex tests will only be allowed in certain laboratories and licensed private clinics where there are suspected cases of inherited and sex-linked diseases. Doctors who perform such tests for any other reason will be debarred and prosecuted.

But opponents of the ban doubt whether the law will be rigorously enforced. "The legislation will simply drive the practice underground and quacks will take over from law-abiding practitioners," says Kiren

Kucheria, who introduced amniocentesis in the early 1970s at the All India Institute of Medical Sciences in Delhi.

Critics point out that, despite the anti-dowry law, some 2,500 brides are murdered every year for not bringing enough dowry. And in Maharashtra state, which banned the SDT in 1988, these tests are still available illegally.

Feminist groups have welcomed the legislation, but they have reservations, arguing that a woman who takes an SDT



should not be punished, as the decision to undergo the test is usually that of her family.

Critics are also unhappy that the law says nothing about the use of technologies for increasing the chances of conceiving a boy.

Meanwhile the killing of baby girls using traditional methods — such as poisoning with plant extracts or putting opium on the nipples of nursing mothers — continues to be used in poor regions such as Tamil Nadu and Rajasthan. According to some reports, about 6,000 baby girls have died of poisoning over the past ten years in one small community in Tamil Nadu. The government is now looking at ways of dealing with female infanticide.

K. S. Jayaraman

Ozone rules prompt dispute over speed limits

Munich. The German state (*Land*) of Hessen introduced the country's first ever motorway speed limits last week in an attempt to reduce tropospheric ozone levels that have reached record levels in central Europe's prolonged period of hot, still weather.

But the speed restrictions — 90 km (58 miles) an hour on motorways, and 80 km an hour on other roads — have caused a political uproar. The federal environment minister, Klaus Töpfer, says that they will have a negligible effect on ozone levels, and describes the action as "politically motivated".

Similarly the federal transport minister, Matthias Wissmann, called the decision "unjustified", an opinion shared by local businessmen who are challenging the actions of Hessen — which, in contrast to the conservative federal government, is run by a coalition of Social Democrats and Greens — in the state constitutional courts.

In common with Germany's other 15 *Länder*, Hessen has the right under the federal law that controls emissions to take steps to prevent the formation of winter smog, including the imposition of limits on traffic speeds. Hessen's environment minister, Joschka Fischer, a prominent member of Germany's Green Party (*die Grünen*) has interpreted this law as applying to summer smog as well.

Winter smog occurs in anticyclones when pollutants, particularly nitrogen oxides, accumulate in a thin layer of cold air trapped by a temperature inversion in which the mixing of air-mass — either horizontally or vertically — is impeded. Anticyclones can also precipitate summer smog through the lack of lateral air movements alone, and in

sunny conditions ozone is produced from the pollutants.

Traffic contributes to concentrations of ozone when NO released in exhaust fumes is oxidized to NO₂. During daylight hours, sunlight splits NO₂ and the released oxygen atoms combine with molecular oxygen (O₂) to form ozone (O₃). If the process takes place during a low exchange of air-mass, ozone can accumulate to levels that are biologically damaging.

Peak ozone levels of more than 215 µg m⁻³ ("first warning levels") were measured at 11 stations in Hessen last week. As no change was forecast in the prevailing weather conditions, the state decided to introduce the speed limits. They were maintained for three days.

According to police reports, 85 per cent of drivers complied with the restrictions on the first day, and most of the rest drove more slowly than usual. Compliance improved still further over the days; normally 17 per cent of Hessen's drivers travel at more than 160 km an hour on motorways.

Peak ozone levels fell significantly after imposition of the speed limits. But Karlheinz Liebl from the Hessen Environment Bureau, which is responsible for the measurements, says that the precise extent to which the speed restrictions contributed to this fall will be known only after detailed analysis of all contributory factors.

Hessen officials are confident that, on the basis of the results of the few studies that have been carried out, speed limits are likely to have been responsible for a fall of between one and three per cent in ozone levels over this period.

Berlin ends medical school clash

Berlin. A fierce dispute over the organization of medical teaching and research in Berlin has ended with the decision to transfer the Rudolf Virchow university clinic of west Berlin's Free University to east Berlin's Humboldt University, where it will be merged with the Charité university clinic.

The transfer will take place next spring, and is the result of a decision by the Berlin government taken after consultation with the universities involved — after months of increasingly ugly wrangling (see *Nature* 369, 431; 1994).

The roots of the conflict lie in the fact that, after reunification in 1990, Berlin found itself with three university medical schools (the third, known until recently as the Steglitz clinic, also belongs to the Free University), which it could not afford to maintain. Under the merger, the total number of teaching-hospital beds in the city will be reduced, and the annual intake of medical students will fall from 1,000 to 600.

Angry at the prospect of losing two-thirds of its preclinical students, the Free University last month embarked on a last-minute publicity campaign to avert the merger.

All teaching staff at the Charité, as in other universities of the former East Germany, have already had to reapply for their jobs as part of a restructuring process and Konrad Seppelt, vice-president for science and research at the Free University, admits that his institution would have readily accepted the merger if it had happened before posts at the Charité had been reaffirmed. But, not surprisingly, the Charité has been offended by the Free University's implication that the Charité should bear most of the staff cuts.

Cornelius Frömmel, the Charité's director of research, also believes that fear of competition from the strengthened Charité could also underlie the Free University's hostility to the merger. **Allison Abbott**

IMAGE
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Germany's transport minister calls restrictions on cars 'unjustified'.

But Töpfer remains unenthusiastic about temporary measures to control ozone levels. He says that serious reductions can be achieved only by reducing production of nitrogen oxides at source. One measure would be the wider introduction of catalytic converters, which reduce output of pollutants by 90 per cent (half of Germany's cars are already fitted, one of the highest rates in Europe).

Another step, he says, would be the introduction of vapour recovery systems in petrol stations to prevent gases from fuel pumps drifting into the atmosphere when cars are being filled. The European Commission has prepared a directive that would require such systems in all forecourt pumps.

Walter Fricke, an ozone expert at the Hessen environment ministry, accepts that such measures are necessary. But he argues that they will take time to introduce. Meanwhile, he says, any measure taken to reduce ozone is important.

The environment minister of neighbouring Lower Saxony has drafted a bill that introduces similar measures to control summer smog. But it goes even further than Hessen. At a second ozone warning level (360 µg m⁻³, the state would forbid the use of any car without a catalytic converter. "We wanted to do this in Hessen, but didn't dare," says Fricke. Hessen is awaiting the outcome of a court challenge to its right to impose speed limits. If it wins, it will consider going the way of Lower Saxony.

But apart from Lower Saxony, which has a Social Democrat government, Fischer has few supporters among the other *Länder*. The environment minister in right-wing Bavaria, for example, called Hessen's action a result of "major hysteria". **Allison Abbott**

Memory of water revisited

SIR — Hirst *et al.*'s claim¹ to have failed to replicate our findings² (that high dilutions of anti-IgE can trigger basophil degranulation) is unfounded as they did not follow our methods²⁻⁷, and we question their statistical analysis.

We have noted fifteen discrepancies in methodology, of which three are cardinal errors: (1) the authors did not eliminate blood that was unreactive to ponderal anti-IgE antiserum, and which self-evidently would not react to the same highly diluted antiserum; (2) they did not test the high dilutions and controls (themselves inadequate, proper controls being diluted dummy antibody) on the same blood samples, an experimental *sine qua non*; and (3) they added a centrifugation step after incubation, thereby impairing accurate counting of activated basophils^{2,5}. These errors alone are enough to invalidate their whole attempt. The procedures involved in our basophil test are complex; it is a pity the authors did not consult us before embarking on it.

Nevertheless, we believe that their data, despite the methodological errors, do support our findings: the differences between active and control sessions are clearly evident in Figs 2 and 3. The authors admit observing those differences, but they seek to explain them away by invoking some "unidentified part" of their procedure. They also try to disguise the differences by some inappropriate statistical analyses: Fig. 1, for example, spuriously combines the high dilution (but not ponderal anti-IgE) and control data, presumably to make the dilutions appear less active. The "comparison within treatment" analysis in Table 1 is weak; had they used the more powerful "comparison between treatments", the true significance of the data would have been revealed. Because of these lacunae, we requested sight of Hirst *et al.*'s raw data, but the authors refused to provide them.

The phenomenon we described² has been confirmed by independent laboratories⁸⁻¹⁰. Our full assessment of Hirst *et al.*'s paper is available on request.

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SIR — We agree with the conclusions of Hirst *et al.*¹. In fact, nearly two years ago our research group published similar results¹¹. We performed 24 blind experiments, of which 14 were independently evaluated by two investigators. No evi-

dence was found for a difference between vortexed and non-vortexed dilutions of anti-IgE (tenfold serial dilutions in the range 10^{21} – 10^{30}) on the staining properties of human basophil leukocytes. In fact, it was concluded that extreme dilutions had no activity at all.

Some differences, however, were found between the investigators in the sense that researcher X counted about 4 more basophils per sample than Y, this being about 5 per cent of the mean ($P < 0.001$ Wilcoxon's test). Although these data are quite reasonable, they indicate that counts do differ between persons. The possibility could therefore not be excluded that there was a vortex-related effect in our experiments that had gone unnoticed. The observation by Benveniste that one person was better at performing positive experiments than others (personal communication) could support this line of thought. Unfortunately, Benveniste never supported an analysis of possible experimenter-dependence in experiments in which someone who normally achieves positive results counts a number of experiments simultaneously with other workers.

Meanwhile, one of the co-authors of ref. 2, Dr Sainte-Laudy, has adapted the procedure of basophil staining that he published nearly three years ago¹². In that procedure, pre-incubation of basophils with ultra-low doses of histamine has been reported to modulate the degranulation triggered by anti-IgE. In this way passive sensitization is avoided. Also, instead of the rather painstaking evaluation of toluidin-stained basophils, another dye (alcian blue) is used which allows rapid and clear-cut basophil counts without time-consuming training. Following this protocol, double-blind experiments are currently being performed in five different laboratories in the United States, Ireland, Italy and France as well as in our laboratory in the Netherlands. These experiments are coordinated by Professor Roberfroid (Louvain, Belgium). The last word has not yet been spoken.

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Proof positive

SIR — I was gratified to note in the 12 May issue (*Nature* **369**, 97; 1994) that John Maddox condemns the use of the phrase "evidence for" when the correct phrase should be "inconclusive evidence for". But I was astonished that on page 164 of the same issue Kevin Davies, the editor of *Nature Genetics*, uses the phrase "... but maybe these are just the exceptions that prove the rule". The context of the article forces one to assume that by these words he means that these exceptions demonstrate that the rule (that intra- and intergenic DNA sequences represent 'junk' DNA) is thus validated. 'Prove' derives from the Latin *probare*, meaning to try, approve or prove, and the first meaning is generally given as 'to test'. Examples are to prove gold, that is, to test its genuineness, or the noun 'proof' as applied to whisky where the test is whether it can be ignited or not. Hence an exception that proves the rule in fact tests the rule and if exceptions are found the rule is false.

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Sophistry . . . ?

SIR — Frank J. Tipler, in his review of *Fire in the Equations* by Kitty Ferguson (*Nature* **369**, 198; 1994) quotes Hume as saying that books that contain neither abstract reasoning on quantity or number nor experimental reasoning on matter of fact and existence can contain only sophistry and illusion. In which category does Hume's statement belong?

Joseph Hertzlinger

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Missing person

SIR — Your report that Sir Martin Rees is to be the new Astronomer Royal (*Nature* **370**, 8; 1994) left a black (or, rather, white) hole where his photograph was expected. Are we to take this as evidence for the suspicion aroused by Star Trek that the task of the Astronomer Royal is to boldly see what no man has seen before?

David Weltzman

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■ The photograph appeared in some editions.

False calculation of π by experiment

An over-charitable investigation of an attempt to calculate the irrational number π by casting a needle at random onto a ruled grid arrives at the conclusion that the experiment was never carried out.

THE irrational number π is inevitably a great source of wonder and a stimulant of speculation. Sadly, perhaps, the sport of adding a few extra digits to the decimal value of π has been killed by computers. But historically, the number embodies the mystery of circularity by relating the circumference of a circle to its diameter (by simple multiplication of the latter). That π is irrational is the origin of the belief that it is not possible to 'square the circle', although a little reflection will show that there is nothing wrong with $\sqrt{\pi}$ except that, like π itself, it is also irrational. What can be objectionable about a square with sides whose length is an irrational number?

In any case, the numerical value of π can be obtained experimentally, so to speak, without ever drawing a circle and measuring its circumference, but simply by the manipulation of straight lines. The first claim to that effect appears to be due to Buffon (strictly, Le comte de Buffon), who pointed out in 1777 that π appears explicitly in the calculation of the probability that, if a straight object such as a needle is thrown randomly onto a flat surface ruled with parallel lines, the needle will intersect one of the lines. The simplest case is when the length of the needle, say l , is less than the separation of the parallel lines, say d , when the probability that the needle will intersect one of the lines is $2l/\pi d$.

From that point on, it was open to anybody to seek a value for π simply by dropping a needle onto a surface ruled with parallel lines set further apart from each other than the length of the needle. This apparently became one of the great intellectual pastimes of the nineteenth century. If N is the number of times the needle is dropped and H the number of times it is found to cross a line, then N/H is an experimental estimate of $\pi d/2l$, giving $2lN/dH$ as the estimated value of π . The most celebrated of the estimates obtained in this way is due to the Italian M. Lazzarini, who announced in 1901 a value of $\pi = 3.1415929...$. In the true value of π , the last digit should be a '6', not a '9', so that the result is accurate to a few parts in 10 million.

Lee Badger, from the Weber State University at Ogden in Utah, evidently shares the view that this result is too good to be true. Writing in the *Mathematical Association of America's* pedagogical *Mathematics Magazine* (67, 83; 1994), Badger describes the result as "lucky". That is a charitable way of putting it. The truth is that if Lazzarini's result had been published in 1994 and not

1901, it would be called a barefaced fraud. Indeed, Badger himself, after elegantly demonstrating that Lazzarini's good luck must somehow have been contrived, himself uses the word "hoax" to describe how an even better approximation to π might be obtained. In short, Badger's tale should be a warning to all those who pollute the literature that their misdeeds will follow them to the grave.

The details of Lazzarini's experiment are to the point. His needle was 2.5 cm long (that is l), his parallel lines were separated by 3.0 cm (d), he dropped his needle onto the marked grid 3,408 times (N) and recorded 1,808 intersections of the needle with a grid-line (H). The exceptional quality of Lazzarini's good luck is easily appreciated: one hit more or less, giving 1,809 or 1,807 rather than 1,808 hits, would have produced a variation of 1/2,000 in the value of π , yielding a departure from the true value in the third rather than the seventh decimal place.

There are other grounds for worrying about the precision of the result, not the least of which are the unavoidable imprecisions in the length of the needle (l) and the spacing between the lines of the grid (d). The obvious difficulty is that an error in either translates directly into a commensurate error in the estimate of π obtained by dropping a needle onto a ruled grid. Would Lazzarini have had access to the metrology equipment that would have allowed his measurements of l and d to be accurate to a few parts in 10 million?

Badger, evidently one in whom the seeds of suspicion arise only with difficulty, puts much the same point in yet another way: why, he muses, should Lazzarini have dropped his needle onto his grid exactly 3,408 times and not, for example, 3,500 times? Is there the possibility, only the slightest possibility, of course, that Lazzarini was guided by his knowledge that the number 355/113 is a rational approximation to π first described as such in the fifth century by a Chinese mathematician?

For as well-mannered a critic of even deceased fellow-beings as Badger, it is evidently distasteful to face up to the enormity of what Lazzarini may have done. The reported dimensions of his experimental equipment nevertheless give the show away. For one thing, the ratio $2l/d = 5/3$. Simply multiplying that by the reported ratio of needle-throws to hits ($3,408/1,808 = 213/113$ after dividing both numerator and denominator by 16) gives the magic ratio 355/113.

But charitable Badger turns the problem around. He allows that Lazzarini may have had the ratio of 355/113 somewhere in mind (and probably nearer the front of it than the back), meaning that the dimensions of his equipment would have enabled him to make a choice every 213 throws of the needle; how good *now* is my approximation to π , he might have asked himself after every 213 throws?

On that view, Lazzarini's reported success would have arisen on the sixteenth attempt, but elsewhere the errant experimental mathematician claims to have cast his 2.5-cm needle 4,000 times. The charitable question is that of the probability that, at some multiple of 213 throws, Lazzarini would have recorded the same multiple of 113 hits. The answer is surprisingly high; the probability that he would have obtained the 'right' answer in eighteen or fewer multiples of 213 throws of the needle is a staggering 0.3, or 30 per cent. Lazzarini may not have been a fraud after all!

Sadly for the memory of the dead, Badger then proceeds to put the knife in. Lazzarini was apparently unwise enough to report not just the number of hits after 3,408 casts of his needle, but also at intermediate values. Sadly, the departure of the reported values from those expected by somebody with a value of π in mind turns out to be consistently smaller than would be expected if the events contrived were random. Indeed, Badger concludes that the chance that the fluctuations from the ideal reported by Lazzarini would be as small amounts to merely 0.000003, or 3 in a million. Unsurprisingly, he concludes that "it seems likely that the experiment was not done".

That, of course, is how those who concoct data are most commonly found out. It is easy enough to ensure that a final result includes a reasonable error, but much more difficult to arrange that the errors in a series of data bear a reasonable relationship to what random processes would yield. And those who have concocted the numbers can always say that the case against them rests "only on probability", as if that were without meaning. Yet, curiously enough, Lazzarini's non-experiment is not without meaning. Indeed, it inspired generations of people to believe that there is indeed a connection between circularity and rectilinear geometry. If it was merely a *gedanken* experiment, it may nevertheless have served what is called a heuristic purpose of some importance.

John Maddox

The progeny of sexual PCR

George P. Smith

FOR about a decade now, molecular biologists have been introducing random mutations into genes in order to probe structure-function relationships and to evolve new functions or improve old ones *in vitro*. The most popular methods include cassette mutagenesis with degenerate oligonucleotides¹ and error-prone polymerase chain reaction (PCR)^{2,3}. These techniques permit construction of large libraries of mutants; and when clones with a particular phenotype can be selected from such a library, the result is a powerful engine for creating desired target functions. But something is missing from the toolkit of applied molecular evolution — something thought to be key for natural evolution. Sex. In reports in *Proceedings of the National Academy of Sciences*⁴ and on page 389 of this issue⁵, Willem Stemmer of Affymax Research Institute addresses this deficiency with a technique I'll call 'sexual PCR'. The first paper describes the technique in detail, the second its application.

Normal PCR is a series of *in vitro* DNA replication cycles, each cycle consisting of a denaturation step that separates base-paired strands, an annealing step that allows primer oligonucleotides to form base pairs with the separated strands, and a polymerization step that allows polymerase to extend the primer, using the complementary strand as template. The descent of the replicating molecules from the initial population can be imagined as bifurcating trees, each branch-point representing separation of two strands at a denaturation step. Ideally, the trees are chastely asexual: separated lineages evolve totally independently of one another. In reality, though, formation of incomplete strands by fragmentation or premature termination during polymerization undoubtedly introduces an element of sexuality. For, during the annealing step, an incomplete strand from one lineage can forge base pairs with a complementary strand from another and subsequently be extended by polymerase, effectively recombining sequence information from the two lineages. Such recombination of genetic material is the essence of sexuality in its most fundamental sense.

In sexual PCR, recombination is promoted artificially by cleaving the initial population of molecules into random fragments, which are then subjected to multiple cycles of PCR without added primer. The term 'chain reaction' is really a misnomer here, because there is no autocatalytic amplification. Rather, incomplete strands become longer and longer as they find complementary strands to template

extension of their 3' ends. Each such interaction is effectively a recombination between different lineages; and if the initial fragments are small, many recombinations are required to complete a strand. Complete strands are then amplified in a second, conventional PCR with added primers. The overall DNA shuffling process is error-prone, so the recombining molecules concomitantly acquire new nucleotide substitution mutations upon which selection can act.

The experiment reported here⁵ dramatizes the power of sexual PCR when coupled with selection. Stemmer started with a β -lactamase gene, TEM-1, with very low activity against the antibiotic cefotaxime; the minimum inhibitory concentration for *Escherichia coli* bacteria carrying a TEM-1-bearing plasmid is only 20 ng ml⁻¹. The gene was subjected to sexual PCR, the PCR product spliced back into the plasmid vector, the spliced vector transformed into *E. coli*, and the transformed bacteria selected on various concentrations of cefotaxime. Several hundred clones showing the highest levels of resistance provided the starting DNA for another cycle of the same four steps. After three such cycles of mutagenesis, recombination and selection, the minimum inhibitory concentration for the most resistant clones was 16,000 times higher than that of the original clone.

One of the most resistant clones, ST-1, turned out to have an up-promoter mutation, four amino-acid replacements and four silent mutations (that is, nucleotide replacements in the coding sequence that do not change the coded amino acid); amino-acid replacements are shown in the table. The ST-1 gene was then subjected to two further cycles of mutagenesis, recombination and selection, with a large

excess of wild-type TEM-1 DNA during sexual PCR; stringent selection was maintained on the output clones from each cycle. Stemmer calls these 'backcross' cycles, because like ordinary genetic backcrosses they select against any of the original mutations that are not important for the selected function.

One of the most resistant mutants produced by the backcross cycles, ST-2, was sequenced. It had indeed lost the four silent mutations of ST-1, but it also gained two new ones. As can be seen in the table, one amino-acid replacement in ST-1, A18V, was reversed in ST-2; but three new replacements also appeared. Was the A18V replacement lost because, like the silent mutations, it is unimportant for the high resistance of ST-1? Evidently not: a deliberately made construct, ST-4, that resembled ST-1 except that it was missing A18V, had a 32-times lower minimum inhibitory concentration (see table). Apparently, then, loss of A18V in ST-2 is compensated by the three new replacements to maintain high resistance.

There are more than 10¹⁹ possible TEM-1 variants with six or fewer amino-acid replacements (the number in the most active mutant, ST-2); Stemmer explored a billion or so at most. Are variants as active as ST-2 then so common that even such a vastly incomplete exploration can find them? A control suggests otherwise: when regular error-prone PCR was used in place of sexual PCR, only a 16-times increase in minimum inhibitory concentration was achieved. Why was sexual evolution so much more successful? In asexual evolution, all new mutations acquired by an evolving DNA molecule are random. In sexual evolution, in contrast, a molecule acquires many of its new mutations by swapping genetic material with other molecules in the population. Because that population has survived selection in favour of the target function, mutations acquired by swapping have a better than random chance of

Selection and sexual PCR

Position	TEM-1 (MIC 0.02)	ST-1 (MIC 320)	ST-2 (MIC 640)	ST-4 (MIC 10)
18	A	V	A	A
42	A	A	G	A
92	G	G	S	G
104	E	K	K	K
182	M	T	T	T
238	G	S	S	S
241	R	R	H	R

Amino-acid replacements in TEM-1 mutants selected for high resistance to cefotaxime. ST-1 descended from TEM-1 through three cycles of mutagenesis/recombination/selection; ST-2 descended from ST-1 through two 'backcrosses'; ST-4 is a deliberately made construct. Arrows show where amino acids in a starting gene have been replaced in a descendant. Minimum inhibitory concentration (MIC) values are in $\mu\text{g ml}^{-1}$.

increasing fitness (the function being selected for — drug resistance in this instance). This argument assumes that mutations that have been selected because they increase fitness in one sequence context will also tend to do so when swapped into another sequence context. The modularity need not be perfect to give sexual selection a distinct advantage.

Sexual PCR requires sequences that are related enough to swap information. The degree of relatedness can be quite modest: using a polymerase that works at low temperature (and had to be replenished in each replication cycle), Stemmer created recombinants between genes whose segments of uninterrupted identity were only 4.1 bases long on average⁴. Still, recombination in a population of random sequences would be restricted to subfamilies of similar sequences — families that would be vanishingly small in the initial stages of

in vitro evolution. If, moreover, the sequences are short, as in random peptide libraries, there may not be enough modularity for sex to be an advantage anyway. So I think the most plausible field of application of the new technique is just the type of project reported here⁵: selection of a new function in an evolving population of related, gene-sized molecules. □

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GALAXY FORMATION

Dust in the distance

R. D. Joseph

THE formation and early evolution of galaxies is one of the fundamental unsolved problems of astrophysics. Theories flourish almost unchecked by observational constraints, because objects seen at early times are very distant and therefore faint. So detecting any property of a galaxy at high redshift is an achievement and is likely to provide a new insight into some aspect of galaxy formation and evolution. One such measurement is reported by Dunlop *et al.* on page 347 of this issue¹. They have detected a signature of dust in the radiogalaxy 4C41.17 — at a redshift of 3.8, the most distant galaxy known.

One of the difficulties besetting researchers in this area is just to find and identify galaxies at cosmological distances. Here, powerful radiogalaxies come into their own, since by virtue of their large intrinsic luminosities they can be detected at great distances. Nearby examples of such radiogalaxies always turn out to be elliptical galaxies, and so high-redshift radiogalaxies might well be the progenitors of present-day ellipticals. A number of astronomers have even argued that this or that radiogalaxy was a 'primaeval galaxy', that is, a galaxy in which the first generation of star formation is taking place^{2,3}. On the other hand, there seems to be considerable diversity of properties among the dozen or so high-redshift radiogalaxies studied⁴ — partly, no doubt, because the high-radioluminosity phase is brief compared with the evolutionary timescale for massive stars.

Dunlop *et al.* chose to study 4C41.17. At a redshift of 3.8 this is the most distant

object yet discovered that has optical properties apparently dominated by light from stars (quasars at greater redshifts are known). Using the James Clerk Maxwell Telescope on Mauna Kea, they successfully detected radiation emitted by 4C41.17 at a wavelength of 800 micrometres. They argue convincingly that this is thermal radiation emitted by dust at a temperature of about 50 K, and they show that this implies a dust mass in the galaxy of about 300 million solar masses. By fitting a typical 'infrared galaxy' spectrum to these data (in the rest frame) they infer an infrared luminosity of at least 5×10^{13} times that of the Sun. This is a prodigious luminosity, rivalling those of the most luminous quasars. Dunlop *et al.* have apparently identified the second-most luminous infrared galaxy known.

The IRAS galaxy F10214+4724, at a redshift of 2.3, retains its title as the most luminous galaxy known⁵, and it is interesting to compare the properties of the two. The radiogalaxy 4C41.17 is a factor of six less luminous and has a third the mass of dust. Measurements of carbon monoxide emission permit determination of the mass of molecular dust in the IRAS galaxy, and imply a gas-to-dust ratio of 400. If one assumes this ratio is applicable to 4C41.17, this gives a mass of molecular gas for the radiogalaxy about a factor of three less than that of F10214+4724. Thus in its infrared luminosity, dust content and inferred gas content, 4C41.17 seems to be less extreme and remarkable than F10214+4724 only by factors of about 3 to 5.

What is the energy source responsible

for the extraordinary luminosities of these two galaxies? The two likely processes are either a burst of star formation or an active galactic nucleus (AGN). There is obviously an AGN in the centre of 4C41.17, but it is certainly not clear that this is the main source of the high-energy photons which are absorbed by the dust and re-radiated in the far-infrared (100–200 μm). Because of the large dust opacity in the centres of virtually all infrared-bright galaxies, it is difficult or impossible to be certain that one can view deep enough to see the highly broadened emission lines associated with an active nucleus if they were present. My own opinion, based on the apparently huge mass of molecular gas in both these objects, is that there is very likely to be a massive burst of star formation going on and that is probably the source of the infrared luminosities. If rapid star formation does cause most of the far-infrared luminosity in either of these galaxies, a star formation rate of 10^3 to 10^5 solar masses per year is required. This is enough to build a large galaxy in 10^7 to 10^9 years.

Are these objects, then, examples of the elusive primaeval galaxies, the Holy Grail of observational cosmology? I would suggest not, for there must already have been one generation of massive stars that have evolved off the main sequence and produced the heavy elements which make up the large masses of dust observed. On the other hand, the masses of molecular gas inferred to be present are a significant fraction of the stellar mass of a giant elliptical galaxy, so what we may be seeing in both cases is a late stage in the formation of a galaxy.

One of the limitations in using high-redshift radiogalaxies to investigate galaxy properties at early times has been that so few had been identified among the vast numbers of radiogalaxies known. Recently, however, it has emerged that selecting those radiogalaxies with an extremely steep radio spectrum is a good way to identify distant galaxies. With a much larger sample of high-redshift radiogalaxies, and the new generation of large telescopes and powerful infrared instruments, their study looks set to become an industry with considerable potential for providing information about the earliest phases of galaxy development. □

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The eleven-year El Niño?

Michael J. McPhaden

THE El Niño event of 1982–83, the strongest of the century, had dramatic effects on the circulation of the tropical Pacific Ocean¹, the marine ecology of the eastern equatorial Pacific^{2,3} and patterns of weather variability around the globe⁴. Its oceanic effects also penetrated to higher latitudes along the west coast of North America where changes in coastal circulation raised sea surface temperatures and led to mass mortality of some fish and marine bird species⁵. But these, it seems, were just the immediate manifestations. On page 360 of this issue, Jacobs *et al.*⁶ suggest that the consequences of the 1982–83 El Niño lasted far longer than anyone had expected.

Jacobs *et al.* used a new generation general circulation model for the global ocean to simulate variability in the Pacific basin between 1981 and 1993. These simulations indicated that collapse of the tradewinds in the equatorial Pacific associated with the 1982–83 El Niño excited a sequence of ocean waves which propagated along the Equator to South America, turned polewards along the west coast of Central and North America, then finally headed offshore into the main reaches of the North Pacific. Comparing the results of this model simulation to global satellite data sets, which show a banded rise in sea level in the North Pacific between 1986 and 1993, Jacobs *et al.* argue that after slowly propagating westward across the basin at mid-latitudes for several years, this ocean wave reached the Kuroshio Extension (a strong, eastward-flowing warm ocean current off the coast of Japan) in the early 1990s. The wave, they say, then pushed the axis of the Kuroshio Extension northwards, producing unusually warm sea surface temperatures across the Pacific from Japan to North America.

Propagation

The sequence of events described is plausible, given our present understanding of the ocean's response to surface wind forcing. Relaxation of the tradewinds in the western and central equatorial Pacific during El Niño is known to excite equatorial Kelvin waves (eastward-propagating planetary-scale waves that are trapped within a few degrees of the Equator). These waves can cross the basin in 1–2 months, then reflect as coastal waves that propagate to mid- and high latitudes along the west coast of the Americas in another 3–4 months. The coastal waves elevate sea level, establishing the boundary condition for the offshore radiation of long Rossby waves, a special class of deep-ocean planetary wave for which the Earth's

rotation is an important restoring force. Rossby waves progress westwards at a speed that is inversely proportional to latitude squared, so that at the latitude of the Kuroshio Extension (35–40° N), it would indeed take several years to cross the basin. Heat transport in the Kuroshio Extension affects sea surface temperature variability in the western north Pacific⁷, so that interannual variations in the position of this strong ocean current, whatever the cause, should be manifested in changes in surface temperature.

Elements of this sequence, then, involving equatorial, coastal and mid-latitude ocean dynamics have been studied previously, although from more limited geographical viewpoints or for shorter periods. But by taking advantage of recent advances in ocean modelling techniques and global satellite data sets, Jacobs *et al.* gain a unique basin-scale, long-term perspective that suggests links between oceanic variations originating in the equatorial Pacific in 1982 and changes in North Pacific sea surface temperature 11 years later.

This study raises several questions. How does the Rossby wave generated by the El Niño, if in fact it is a real feature in the ocean, survive so many years as a detectable signal at the latitude of the Kuroshio Extension? Rossby waves have been observed to cross the basin in the aftermath of El Niño events in the tropics, but there they have much higher westward propagation speeds⁸. At 35–40° N, on the other hand, nonlinear interactions, dissipation due to ocean eddies and scattering from the irregular topography of the sea floor would have longer to act on more slowly propagating waves, tending to reduce their amplitude and spatial integrity. In addition, there is considerable seasonal and annual variability in surface wind forcing in the North Pacific⁹. One would expect the oceanic response to this forcing to obscure Rossby wave signals from the west coast of North America. Perhaps, though, this particular wave survives as a coherent, observable signal because it was generated by an unusually strong El Niño. If so, then other weaker El Niños such as those in 1986–87 and 1991–93 are unlikely to produce long-lived or robust Rossby wave signatures north of 30° N.

The wave processes described by Jacobs *et al.* should in theory apply equally well to the Southern Hemisphere. Available data indicate that the first stage was there: poleward wave propagation at low latitudes along the west coast of South America was seen in response to the 1982–83 El Niño¹⁰, which could establish a necessary boundary condition for the offshore radia-

tion of Rossby waves at higher latitudes. Is there evidence for a Rossby wave like that in the Northern Hemisphere, affecting current structures and sea surface temperatures at mid-latitudes in the early 1990s? Examination of ocean temperature and satellite altimetry records could reveal a few surprises.

Impacts

Perhaps the most important issue raised by Jacobs *et al.* relates to the broader climate impacts of the sea surface warming that may have resulted from a shift in the axis of the Kuroshio Extension. Attempts to relate climate variations over North America to mid-latitude sea surface temperature anomalies date back at least four decades¹¹. Typically, atmospheric teleconnections between the tropics and higher latitudes during El Niño lead to an intensification of the Aleutian Low pressure centre and a southward displacement of the jet stream in the North Pacific, altering weather patterns primarily in the winter season over North America^{12,13}. Simultaneously, greater air–sea heat exchange and ocean mixing associated with storms steered by the jet stream produce a broad region of surface cooling in the interior North Pacific¹⁴. Warming off the west coast of North America is also typically observed during El Niño, through a combination of both oceanic and atmospheric processes. But it is unclear how much these temperature anomalies feed back on the overlying atmospheric circulation^{15,16}.

The contemporaneous effects of the 1991–93 El Niño on mid-latitude sea surface temperatures are evident in Fig. 1 of Jacobs *et al.*, namely atmospherically forced ocean cooling in the central Pacific between latitudes of 20–35° N, and warming off the west coast of North America. However, according to Jacobs *et al.*, warming to the north of 35° N in the interior ocean in 1991–93, presumably due to northeastward advection of warm water in the Kuroshio Extension, is a consequ-

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ence of the 1982–83 El Niño, rather than of the 1991–93 El Niño. Whether this particular pattern of surface temperature anomalies has a strong feedback to the atmosphere remains to be demonstrated.

In this regard, though, it is interesting to note that the most dramatic meteorological event over North America during 1993 was the record-breaking summer rainfall and flooding of the Mississippi river basin¹⁷. Was this flooding, the worst of the century in the central United States, a delayed effect of the strongest El Niño of the century? Sensitivity experiments using

atmospheric general circulation models, forced by observed Pacific sea surface temperatures, may provide clues. Equally important will be independent corroboration of the decadal long-ocean-wave sequence and its effect on North Pacific sea surface temperatures from other kinds of ocean general circulation models and from *in situ* data sets. □

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GENETICS

Psi no more for yeast prions

M. F. Tuite

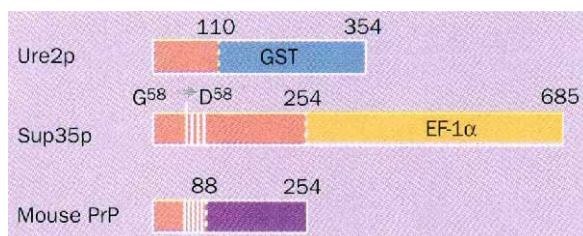
CELL biologists often look to the yeast *Saccharomyces cerevisiae* as a convenient model system in which to study universal problems of eukaryotic cellular physiology and gene regulation. In a reversal of this situation, three recent papers^{1–3} look to work on mammalian neurodegenerative disease to explain a long-standing genetic puzzle in yeast. They invoke the 'prion' hypothesis⁴ originally proposed to explain the transmission properties of the mammalian spongiform encephalopathies, such as sheep scrapie, bovine spongiform encephalopathy (BSE) and human Creutzfeldt–Jakob disease, to explain the strange properties of some extrachromosomally inherited determinants in yeast.

Yeast provides several examples of what are loosely called 'extrachromosomally inherited determinants', controlling phenotypes that appear to be genetically based but are inherited in a non-mendelian manner. The physical basis for some of these determinants is well established — the *petite* phenotype, for example, is due to mutation in the mitochondrial genome. But the underlying genetic basis for other determinants remains elusive.

The two determinants in question are [*URE3*] and [*psi*]. Despite many years of study, no episomal nucleic acid had been found for either. The new work now suggests that both determinants may be manifestations of nuclear-encoded proteins able to exist in two different conformational (and presumably functional) states, by analogy with the mammalian prion proteins (PrPs).

The [*psi*] determinant was fortuitously uncovered some 30 years ago as a modifier of tRNA-mediated nonsense suppression in yeast that was inherited in a non-mendelian fashion⁵. The [*psi*]-associated

phenotype is simple: in a [*psi*⁺] cell nonsense suppression is efficient, whereas in a [*psi*[−]] cell this efficiency drops some ten-fold. That there is a non-nuclear determinant defining the [*psi*] status of a cell has been confirmed by all conceivable genetic means⁶, yet no cytoplasmic nucleic acid presented itself as a candidate.



Organization of the yeast prion proteins Ure2p and Sup35p and of the mouse prion protein PrP. The locations of the peptide repeat sequences in the N-terminal domains of Sup35p and mouse PrP are indicated, as is the *PNM* allele mutation (*G*⁵⁸→*D*⁵⁸) in Sup35p. The locations of known homologies in the C termini are indicated: GST, glutathione-S-transferase and EF-1α, elongation factor 1α. Numbers refer to the numbering of amino-acid residues.

The realization that [*psi*] might have a non-nucleic acid determinant came with the discovery that a [*psi*⁺] cell is converted permanently to a [*psi*[−]] cell by growth for several generations in the presence of a protein-denaturing agent such as guanidine hydrochloride⁷. The [*URE3*] determinant can be similarly 'cured' by protein-denaturing agents¹.

The case for a prion-like analogue underlying the [*URE3*] determinant has been demonstrated by Wickner¹, who proposed, on the basis of a series of elegant genetic experiments, that the [*URE3*] phenotype — the ability to utilize ureidosuccinate in the presence of ammonium ions⁸ — is the result of a conformational change in the product of the nuclear gene, *URE2*. This protein (Ure2p) negatively regulates nitrogen metabolism and has significant amino-acid identity with glutathione-S-transferase (GST)⁹.

Following the proposal by Wickner that [*psi*] may, like [*URE3*], have a prion-like protein as its determinant¹, two papers in the July issue of *Genetics*^{2,3}, point the finger convincingly at the *SUP35* gene as encoding the underlying protein determinant of the [*psi*] phenotype.

The product of the *SUP35* gene (Sup35p) is a 685-amino-acid protein with a carboxy terminus showing striking homology to the translation elongation factor EF-1α (refs 10, 11). Consistent with the [*psi*]-associated phenotype, a number of mutations in the *SUP35* gene have been identified that modify the efficiency of tRNA-mediated nonsense suppression¹². The significance of the two new papers is that they show that point or deletion mutations in the *SUP35* gene can result in permanent abolition of the [*psi*⁺] phenotype. Doel *et al.*³ describe a dominant allele (the *PNM*, or *psi*-no-more allele) with a single point mutation (glycine to aspartate) in the amino terminus of Sup35p. This, together with the [*psi*[−]]-inducing *SUP35* deletion mutations described by Ter-Avanesyan *et al.*², shows that the N terminus of the Sup35p protein is essential for maintenance of the [*psi*⁺] phenotype. Unlike the *PNM* mutant,

however, the N-terminal deletions described by Ter-Avanesyan *et al.*² are genetically recessive. Furthermore, a [*psi*[−]] strain carrying an N-terminally deleted *sup35* allele can maintain [*psi*⁺] by overexpression of the N-terminal 114 amino acids of Sup35p in *trans*².

Scrutiny of the N terminus of Sup35p reveals the existence of several tandem nonapeptide repeats with the consensus PQGGYQQYN (V. V. Kushnir, I. Stansfield and B. S. Cox, personal communication). Intriguingly, the mammalian PrP proteins also have, at their N

termini, up to five tandem octapeptide repeats with the general consensus PHGGGWGQ⁴, a repeat sequence not dissimilar to the Sup35p repeat. Although Ure2p has no such repeats, Ure2p, Sup35p and mammalian PrPs all show related predicted secondary structural features at their N termini — an almost complete lack of α-helices and β-sheets, but a high density of turns (V. V. Kushnir and I. Stansfield, personal communication). The importance of the Sup35p repeats for [*psi*⁺] maintenance is confirmed by the *PNM* mutation, which results in a non-conservative amino-acid replacement of the second glycine residue within the first nonapeptide repeat³.

A further property shared by the yeast prions and their mammalian counterparts is the consequence of overexpression of the wild-type PrP gene. Overexpression of mouse PrP in transgenic mice results in the

appearance, in older mice, of disease akin to a prion-based disease¹³. Similarly, the overexpression of the *URE2* and *SUP35* genes both result in the *de novo* appearance of the corresponding [*URE3*] and [*psi*⁺] phenotypes^{1,14}.

A recent conformational model of prion replication¹⁵ might equally well apply to the two yeast prion-like phenomena. This model invokes the existence of three conformationally distinct forms of the PrP: the wild-type form (PrP^C), and a partially unfolded monomer (PrP^{*}), which is an intermediate in the formation of the 'infectious' form, PrP^{Sc} (ref. 15). PrP^{Sc} would act as a template to chaperone the conversion of PrP^{*} into PrP^{Sc}, the formation of PrP^{Sc} being irreversible because of its insolubility. The [*psi*⁺] and [*URE3*] phenotypes may therefore represent the PrP^{Sc}-equivalent states of Sup35p and Ure2p respectively.

The new findings with Sup35p imply that the N-terminal domain, and particularly the repeats within this domain, may be important for establishing the PrP^{Sc} state in the yeast prions. It is striking that for both Ure2p and Sup35p, the N-terminal domains are followed by a domain sharing significant identity with a protein of known function: for Ure2p, glutathione-S-transferase, and for Sup35p, EF-1 α . Could these phenomena hint at a more general mechanism used by cells to regulate the biological activity of a protein by altering its conformation using an N-terminal 'switching' domain? It will be interesting to see how many other proteins share this organizational feature.

Identification of the underlying determinants of these 'extrachromosomal' genetic phenomena in yeast will provide us with clues to prion formation and transmission, and demonstrate the importance, across evolutionary time, of a novel biological mechanism. □

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Evolution of water on Mars

Bruce M. Jakosky and John H. Jones

THE martian surface has geological features that suggest that the climate has changed substantially during its history. High erosion rates and the presence of valley networks on the older geological units — some seen in the photograph below — are usually interpreted as signs that abundant liquid water was present before about 3.5 billion years ago^{1,2}. Quantitative clues as to the evolution of the atmosphere, and its water content in particular, can be inferred from various systems of stable isotopes in the martian environment. Last month in *Science*, measurements by Watson *et al.*³ of the deuterium-hydrogen ratio in water derived from the SNC meteorites (convincingly thought to be from Mars) provided valuable new clues to the history of water on the planet.

To be specific, they find that the D/H ratio is enriched up to five times the terrestrial value in mineral grains in the meteorites (see figure), mirroring the enrichment of D/H in the martian atmosphere measured spectroscopically from the Earth^{4,5}. So high an enrichment does not occur on Earth, strongly suggesting that the D/H ratio of the martian atmosphere was established by loss of atmospheric hydrogen to space after the planet's formation³⁻⁵. The magnitude of the enrichment and its similarity to the martian atmosphere suggests that there has been an effective mechanism for mixing the atmospheric water to some depth within the martian regolith, and that the water in these meteorites is directly derived from or has equilibrated to varying degrees with water in the atmosphere. The most plausible mixing or exchange mechanism may be through hydrothermal systems associated with intrusive volcanics or with large

impact craters.

If hydrothermal systems really existed on Mars, they would undoubtedly have affected the evolution of water in the atmosphere. Most directly, they would provide an extremely large reservoir of water that could exchange with the atmosphere. To judge from the volume of water erupted to form the observed catastrophic flood channels, the martian crust might have contained enough water to form a global layer 500 m thick or greater². The total volume of martian extrusive volcanics is equivalent to a global layer of basalt at least half a kilometre thick, and including intrusive volcanism would yield a global layer about 3 to 6 km deep⁶. Heat from large impacts also could have provided a substantial thermal source for hydrothermal systems⁷. Clearly, there are both abundant thermal drivers and large amounts of water available for participation in hydrothermal systems. There is some geological evidence for martian hydrothermal systems, in the form of channels present on some of the large volcanoes⁸; there is some, too, in the SNC meteorites⁹, although the systems have not been previously recognized to exchange volatiles with the atmosphere. In addition to exchange via hydrothermal systems, the catastrophic outflow channels are clear evidence that water has in the past been released from the subsurface and flowed over the surface, where it may have exchanged with the atmosphere¹⁰.

Given the similarity of the D/H ratio in the atmosphere and in the water in the SNC meteorites, the atmosphere probably provides the primary conduit through which water exchanges between many of the reservoirs (see ref. 11 for more details of the different aspects of the martian

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Martian mosaic — traces of ancient river channels in Viking images of the planet.

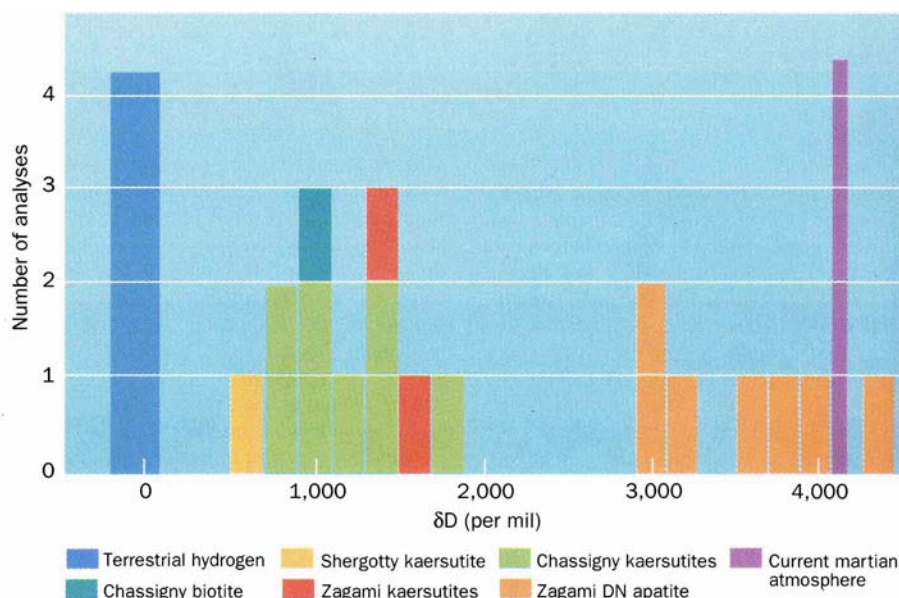
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system). At present, atmospheric water vapour can condense onto the polar caps or sublime from the north polar cap during the summer, and the residual caps contain a large amount of water (and possibly carbon dioxide) as ice. Both water and CO₂ can mix to the upper atmosphere, where the individual atoms can be lost to space; understanding these loss processes is important in unravelling the history of water, nitrogen and CO₂, as well as of the light noble gases neon and argon¹².

The enormous degree of deuterium enrichment observed can only be attributed to the escape of hydrogen to space, as the lighter H isotope escapes so much more readily than D (refs 4, 5). That the water incorporated into the SNC meteorites shows this same enrichment requires exchange between atmospheric and volcanic water. Getting the water from the atmosphere into the deep regolith may be problematic, given the inability of liquid water to exist today at or near the surface except under special circumstances; the new evidence, however, seems to be telling us that it has happened, and that evidence looks sound.

The large spread of D/H values shown in the figure most probably represents varying degrees of exchange or isotopic equilibration of meteoric water either with the magma or with the crystallized minerals themselves³. To judge from the ages of the rocks, D/H must have been set before about a billion years ago; and from the spread of ages, it cannot be evolving rapidly at the present. In contrast, the very presence of neon and helium in the atmosphere, combined with their short lifetimes for removal to space^{12,13}, argues for the continued outgassing of juvenile gases to the atmosphere in the recent geological past.

If meteoric water has been exchanging with the mantle-derived material, and essentially mixing the atmospheric and volcanic water together, then this mechanism also may represent a means for resetting or buffering the atmospheric oxygen isotopes. Fractionation of oxygen can occur through nonthermal escape from the atmosphere to space, again with preferential escape of the lighter isotope¹². On the Earth, exchange through hydrothermal systems at mid-ocean ridges plays an important role in determining the oxygen isotope ratios of the Earth's oceans¹⁴. But actual measurements of the martian oxygen isotopes, in water derived from the same meteorites, are confusing¹⁵. Those data seem to indicate that the oxygen in water is isotopically distinct from that in the rock, and that it was incorporated into the rock without having equilibrated with it. Without a precise measurement of the oxygen isotopes in the martian atmosphere, however, the exact degree of equilibration is uncertain. It might not be surprising that



Histogram of the δD values of water obtained from the SNC meteorites, compared with terrestrial values and the present martian atmosphere. δD is a measure of the D/H ratio in the sample, and is defined as $\delta D = \{[(D/H)_{\text{sample}}/(D/H)_{\text{standard}}] - 1\}$ per mil. Values are reported relative to that of standard mean ocean water. Figure adapted from ref. 3.

the atmosphere ultimately controls the D/H in the rock while the rocks control the oxygen isotopic ratios in the atmosphere — the rocks contain much more oxygen than they contain hydrogen.

If martian hydrothermal systems that are capable of exchanging volatiles with the atmosphere exist, then they would bring water, energy and dissolved minerals close to the surface. Such environments on the Earth are where life thrives at the present and where it might have begun. The martian environment seems otherwise inhospitable, and there is a serious question of whether life-sustaining processes could have occurred on Mars. Thus, could they be found, active or previously active near-surface hydrothermal systems might represent plausible locations to look for extant or extinct life¹⁶.

Clearly the martian water (and by implication the entire climate system) represents a much more complex system than we have imagined. Sinks for water over geological time include the polar caps, ice and bound water in the regolith, escape to space, and interaction to an unknown extent with the regolith, possibly through hydrothermal systems. The current measurements are insufficient to let us sort out the magnitude of each sink or to understand uniquely the evolution of the martian climate and volatile system. New measurements of the martian volatile system will be necessary for a deeper understanding. Of most value would be direct measurements of the effects of escape to space (the current escape rates of oxygen, hydrogen, nitrogen and carbon, as well as neon and argon) and their effects on the isotopic ratios of the remaining atmosphere; a suite of samples re-

turned from known locations which could provide valuable data on the ages of geological events and the history of interactions with the atmosphere; and direct measurements of the isotopic ratios of all of the gases in the martian atmosphere, which would let us pin down possible evolution processes. In the meantime, continued examination of the SNC meteorites and integration of the results with other Mars data (including those from the forthcoming Mars Global Surveyor mission) may provide the most new insights into martian volatile evolution. □

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Solving the specificity puzzle

Graham R. Williams

THE molecular structures of $1\alpha,25(\text{OH})_2$ -vitamin D_3 (D_3) and 3,5,3'-L-triiodo-thyronine (T_3) are so different that nobody could predict their remarkable functional relationship. That was before 1986 and 1987 when the D_3 and T_3 receptors (VDR, T_3R) were respectively cloned and recognized as members of the ever-expanding nuclear receptor superfamily. Since then, mechanisms underlying D_3 - and T_3 -dependent activation of gene expression have been shown to be increasingly hierarchical, involving the addition of multiple control steps to maintain specificity (see table).

Heterodimerization with receptors for 9-*cis* retinoic acid (RXR) has been considered central to VDR and T_3R activity, and an intriguing new piece of this complex mechanistic puzzle is provided by Schröder *et al.* on page 382 of this issue¹. They demonstrate that VDR/ T_3R heterodimers have an independent discriminatory role that is dictated by the target DNA-binding site. Thus, response-element-dependent anisotropic heterodimer binding determines whether D_3 or T_3 transactivation occurs (see figure). VDR/ T_3R heterodimers now must be placed alongside RXR-containing heterodimers as major determinants of vitamin D and thyroid hormone signalling.

The VDR, T_3R and retinoic acid receptors (RARs) activate target genes after binding as homodimers or heterodimers to response elements containing core AGGTCA hexameric sequences arranged in a direct repeat. These observations were the first to raise the question of how receptor specificity is maintained in the face of apparent promiscuity, and led to the notion that spacing of 3, 4 or 5 nucleotides between direct repeat hexamers determined whether an element was responsive to D_3 , T_3 or retinoic acid². RXR was later characterized as a universal partner for each receptor, resulting in the formation of heterodimers which obeyed the 3, 4, 5 rule³. Because of the almost ubiquitous expression of RXR⁴ and the high DNA-binding affinity of RXR-containing heterodimers⁵, these complexes were considered to be the most important mediators of hormone action. Multiple T_3R , RXR and RAR genes and isoforms added another level of complexity to control specificity.

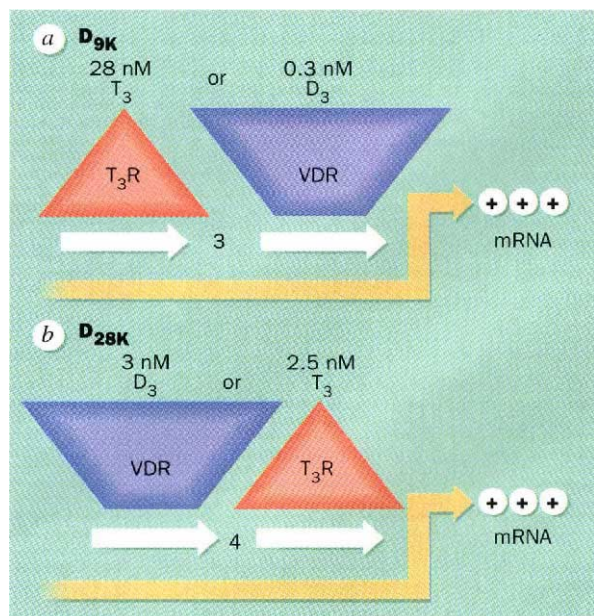
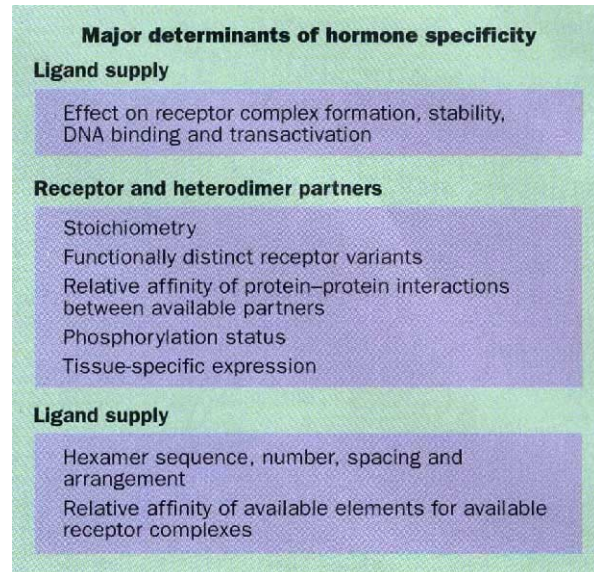
All seemed well at this stage, but several surprises followed. Naturally occurring response elements often contain hexamers of divergent sequence arranged in variable configuration. So the 3, 4, 5 specificity rule², based on hexamer spacing, must be extended to take hexamer sequence and orientation into account.

Studies demonstrating consistent polarity of RXR-containing heterodimers indicate that response-element interactions are also constrained by the relative orientation of receptor DNA-binding and dimerization domains. Such constraints were predicted to influence transcriptional responses^{5,6}, and are imposed by RXR which occupies the up-stream hexamer relative to its heterodimer partner. However, homodimerization of VDR (ref. 7) and T_3R (ref. 8) on elements of variable configuration^{5,7,8} indicates that these molecules are highly flexible⁸ and exhibit the potential for increased promiscuity.

Schröder *et al.* now bring new data to bear on these arguments. Polarity of VDR/ T_3R heterodimer binding is determined by precise hexamer sequence, spacing and arrangement in both direct repeat and inverted palindrome elements. The consequences are remarkable. VDR/ T_3R heterodimers can be activated by either ligand, but only the 3' situated receptor is activated by low hormone concentration. A ten times higher concentration of ligand is required for induction when its receptor is located 5'. In this model, hormone supply to the nucleus is an additional determinant of VDR/ T_3R transactivation.

These observations throw up further questions and a number of inconsistencies. Unliganded T_3R (and probably VDR) homodimers act as transcriptional repressors. Addition of hormone results in homodimer dissociation from certain, but not all, response elements. It was therefore proposed that hormone activation is a two-stage process of de-repression, occurring when aporeceptor homodimers are dissociated, followed by hormone activation of heterodimers⁹. In contrast, 9-*cis* retinoic acid promotes RXR homodimer formation, resulting in activation of RXR-dependent path-

ways and repression of D_3 and T_3 responses, possibly by sequestration of RXR^{10,11}. Importantly, whereas the presence of unliganded RXR increases the binding and activity of VDR and T_3R ^{3,7,9,11}, combinations of ligands may result in additive¹, synergistic⁷ or repressive^{10,11} effects on heterodimer function that seem to be response-element specific. In the new studies¹, 9-*cis* retinoic acid did not modify VDR/ T_3R heterodimer function but the ratio of VDR to T_3R did. Furthermore, co-expression of RXR



Anisotropic binding of VDR/ T_3R heterodimers to wild-type response elements from calbindin $\text{D}_{9\text{K}}$ (a) and $\text{D}_{28\text{K}}$ (b) (relative molecular masses 9,000 and 28,000, respectively) target genes. Elements contain two hexamers in a direct-repeat configuration separated by 3 or 4 bases respectively, one preferentially binding T_3R (red), the other VDR (blue). Induction of transcription is preferentially mediated by the receptor situated in the 3' position. Ten times lower concentrations of D_3 , or ten times higher concentrations of T_3 , are required to transactivate the $\text{D}_{9\text{K}}$ element relative to the $\text{D}_{28\text{K}}$ element.

resulted in ligand-independent squelching of the hormone response, suggesting that receptor stoichiometry is a critical determinant of responsiveness.

It is not easy to summarize the current standing from available data. The actions of D_3 and T_3 can be mediated by VDR or T_3R homodimers^{7,8}, VDR/ T_3R heterodimers¹ or RXR-containing heterodimers^{3,5,7}. Hormone responses are specified by a dynamic equilibrium of many factors including intranuclear concentration of ligand, stoichiometry of co-expressed receptors and the specific response-element sequence activated. The attractive findings presented by Schröder *et al.*¹ elegantly show how variation of these parameters can provide a combinatorial control system of almost limitless variation. It will be interesting to see if this model of specificity is applicable to other signalling pathways.

We now understand many mechanisms whereby information is transferred from hormone to target gene, but we remain in the dark as to how transcription is actually activated. All the data so far have been derived *in vitro*. Assembling the solution

to the specificity puzzle in molecular terms provides a platform. The leap forward will be to determine what heterodimers are formed *in vivo* and what hormone interactions are physiological. Only then will the implications of the surprising relationship between such unlikely partners become clear. □

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ARCHAEOLOGY

The last people alive

Jared M. Diamond

AMONG the most tantalizing mysteries of archaeology are the unexplained disappearances of human populations isolated on islands. The example closest to Europe is that of the Greenland Norse at some time in the late fifteenth or early sixteenth century, but human populations also met mysterious ends on over a dozen Polynesian islands, including Pitcairn Island long before the mutineers of HMS *Bounty* arrived.

Marshall Weisler¹ now helps to resolve this mystery for Pitcairn's neighbour Henderson Island, and by inference for Pitcairn itself. Weisler's study extends the concept of minimum viable population, applied to animal populations by conservation biologists², to humans. His work also tests our understanding of human group dynamics — and tears at the heart-strings with its portrait of a tiny human population dying under the social equivalent of solitary confinement.

Pitcairn and Henderson, 180 km distant, are among the world's most remote habitable islands. Pitcairn is a high extinct Pleistocene volcano only 4.5 km² in area. Henderson is a larger (36 km²), flat, raised coral atoll, but is more marginal for human settlement because it lacks permanent fresh water, has no volcanic rock and offers only small areas of thin soil. Nevertheless, the *Bounty* mutineers found evidence of a former Polynesian settlement

on Pitcairn, and subsequent archaeologists did the same on Henderson. Why did ancient Polynesians settle such remote islands at all, and why did they eventually vanish?

Weisler's evidence consists of ¹⁴C-dated sites with abundant human artefacts, house floors and postholes, human skeletons, tens of thousands of bones of animal prey, and remains of gardens. It emerges that Polynesians colonized Henderson by AD 1000, probably from Man-

gareva Island over 400 km to the west. Henderson's attractions were its previously untapped resources of fish and shellfish, large colonies of nesting seabirds, flightless or weak-flying pigeons and other landbirds, and nesting sea turtles. Polynesians added to these natural resources by importing crops and useful plants, including coconut trees, swamp taro (*Cyrtosperma*), candlenut trees, banana, the shrub *Cordyline fruticosa* for thatching, food and clothes, and possibly pandanus trees and sweet potatoes. Scantier archaeological evidence suggests that Pitcairn, which is much more suitable for farming than Henderson, was occupied around the same time.

Henderson's resident population may have numbered barely 50 people, Pitcairn's perhaps a few hundred. Nevertheless, for several centuries the two populations remained in contact with each other and with the parent society in Mangareva. Henderson's sought-after exports would have been luxury goods: turtles, prized as a prestige food for Polynesian chiefs, plus seabirds used for food and feathers. Pitcairn's export was high-quality volcanic stone. Imports into Henderson included raw materials for manufacturing tools: black-lipped pearlshell from Mangareva to make fishhooks, and several types of stone (volcanic glass, fine-grained basalt and scoria) from Pitcairn to make adzes and other cutting and abrading tools. Other imports would have been pigs and ovenstones (and maybe marriage partners).

As indicated by the eventual disappearance of the imported materials on Henderson, its interactions with Mangareva and Pitcairn ceased around AD 1450. The cause lay far from Henderson: Mangareva's expanding human population deforested and eroded the Mangareva landscape. Pitcairn also became eroded, while many of Henderson's landbirds and some of its seabirds were extirpated³, and its

IMAGE
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Inhospitable terrain — limestone formations of the North-West Pinnacles on Henderson Island. The fissures, which are about a stride across, are as much as 2–3 metres deep.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Pitcairn islanders, all descendants of the mutineers of HMS *Bounty*, in front of the *Bounty's* anchor. The mutiny occurred in 1789, and after setting Captain Bligh and others adrift the remaining nine crew members sailed to Pitcairn. There they settled, and remained undetected until 1808.

turtle population was overharvested. Mangareva's human population survived but descended into an orgy of war and cannibalism that made continuation of trade with Pitcairn and Henderson untenable.

After contact with Mangareva and Pitcairn came to an end, Henderson islanders continued to survive in isolation for about 150 years, or some six generations. Without any volcanic stone as raw material, they learned to fashion adzes from giant clam shells, ovenstones from limestone cobbles and fishhooks from inferior local pearlshell. But without trees large enough to build canoes for open-ocean voyaging, the islanders had no means of getting in touch with the outside world. At some point around AD 1600 the last humans on both Pitcairn and Henderson died.

Why did the populations die out? Speculation on that question can be informed by what happened in similar situations in modern times. Eventually, no potential marriage partners could have remained who did not violate incest taboos, as in the case of California's last four Yahi Indians⁴. Deleterious effects of inbreeding may have appeared before that, as seen for the Pennsylvania Amish, the inhabitants of Swiss mountain valleys, and many other isolated human populations. Climatic fluctuations in an already marginal environment may have driven the islanders to starvation, as happened to Japan's garrison stranded on Wake Island during the Second World War. The people of Henderson may have responded to that threat by murder and cannibalism, like so many human groups under similar conditions: Mangareva Islanders, Easter Islanders⁵, and California's notorious Donner Party of pioneers⁶. And the islanders may have become insane from social deprivation, as happened to members of the Belgian Antarctic Expedition trapped by ice for over a year⁷.

Michael Brooke

Even had the islanders somehow avoided all of these fates, they would still have run up against the problem that 50 people are too few to constitute a viable human population. All plant and animal species have so-called minimum viable populations for demographic reasons². Minimum viable populations must be especially large for gregarious species, whose social structure is essential for reproduction and cultural transmission. Humans are such a species. Studies of human populations stranded in the midst of plenty on Flinders, King and Kangaroo Islands show that even a society of sever-

al hundred people is insufficient to propel human culture indefinitely^{8,9}. Greenland's Norse population became hopelessly conservative, refused to adopt the successful subsistence methods of their Eskimo neighbours, failed to invent equivalent methods themselves, and died out within a few generations of the visit of the last ship from Norway¹⁰. Aboriginal Tasmania's 5,000 inhabitants did survive in isolation for ten millennia; but they abandoned such useful practices as bone-tool manufacture and fishing, and failed to invent the spearthrowers and other devices developed on the Australian mainland⁹.

Today, as hundreds of years ago, the Pitcairn islanders' well-being depends on contact with the outside world, as the remaining 56 descendants of the *Bounty* mutineers look increasingly to supply ships from New Zealand for sustenance in the form of canned and frozen food, and for culture in the form of videos. But to paraphrase Rhys Jones's comment about the Flinders islanders, small societies may die out not only from a lack of food, but also from a lack of people⁸. □

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DAEDALUS

Sex in the head

ARE men and women equally bright, or bright in the same way? One school of thought holds that men and women are identical in all respects, or if they aren't they should be, and we must appoint women extensively to scientific, technical and military jobs to prove how earnestly we believe it. Another school claims that feminine intuition is at least as valid as masculine logic, and that feminist sciences and mathematics should be set up to compete with the male varieties. Daedalus now proposes a crucial test. He plans to abolish testosterone from the male brain.

Testosterone, the male hormone, gives men their male physical and putative mental character. It is claimed to underpin their interest in conquest and mastery — of women, rivals, materials and territory. Science, for example, is the conquest and ownership of intellectual territory (which is why priority squabbles between scientists are so bitter). Daedalus wants to stop testosterone crossing the male blood-brain barrier, and see what happens.

To pass this barrier, testosterone has to escape from the big protein molecules that normally carry it. So DREADCO's biochemists are injecting a special coupling agent into the bloodstreams of male volunteers. It binds testosterone firmly to its protein. The hormone still gets round the body, maintaining male physique, beard growth and so on. But it loses all access to the brain.

A man on DREADCO's 'Neuterminde' will still be completely male. But his brain won't know it. He will be unaware of sexual desire or competitive challenge. Only direct physical assault will arouse him to sex or aggression. He will then react by blind, spinal-cord instinct — thus revealing what these basic human instincts are. Afterwards he will truly 'not know what came over him'. But Daedalus is most interested in his volunteers' mental life. Freed from the burdens and biases of manhood, will they develop childlike insights and subtle illogical intuitions? Will they find feminist algebra suddenly convincing? Most interesting of all, will their scientific creativity blossom or fade? Freud, who saw both art and science as sublimated sexuality, would predict the latter. Without the spur of testosterone, he would say, they simply won't bother to go looking for trouble, mental or physical.

Neuterminde could be useful too. As a depot drug, it should turn male criminals into placid, unadventurous citizens. And it would be the perfect way to tell art from pornography. Take a dose of Neuterminde, and if it's still interesting, it's art.

David Jones

Mammoth DNA sequences

SIR — Lindahl points out¹ that the study of ancient DNA requires stringent criteria for establishing the authenticity of sequences, particularly as some claims seem to contradict chemical knowledge about diagenetic processes^{2,3}. In addition to criteria suggested previously⁴, Lindahl suggests that sequences should be verified by reproduction from different individuals of a species, that negative results should be reported along with the positive ones,

individual yielded identical sequences.

In the figure at the foot of the page, the sequences from the mammoths are aligned to the homologous sequences of the two living members of the order Proboscidea, the Indian (*Elephas maximus*) and African (*Loxodonta africana*) elephants, and to two other ungulates. Whereas no insertions or deletions have occurred among the mammoth and elephant sequences, three and four insertions/deletions are needed for an alignment to the cow and horse sequences, respectively. Among the 69 positions where all sequences are unambiguously alignable, the mammoths differ by 0–4 substitutions from the elephants and by 6–12 substitutions from the other ungulates. Thus, the sequences from the mammoth carcasses are not identical to, but are clearly more closely related to, elephant than to the other ungulate sequences presented here. That different but closely related sequences are retrieved from different mammoths further supports the conclusion that they are of ancient origin.

The mammoths differ from each other by 0–5 substitutions whereas the Indian and African elephants, representing two genera, differ by only two substitutions. These preliminary data indicate that the mammoths were highly diverse and may have been divided into geographical or temporal subspecies.

Thus, Pleistocene DNA sequences fill-

filling the criteria suggested by Lindahl¹ and others⁴ can be retrieved. We suggest that the much older sequences reported in the past few years, for example in amber^{7–9}, be submitted to the same kind of tests as the faunal remains presented here.

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ORIGIN OF FIVE SIBERIAN MAMMOTH SPECIES				
Specimen	Age (yr)	Samples		Ref.
		Total	Positive	
Yuribei (1979)	9,700	4	3	10
Dima (1977)	40,000	2	1	11
Sanga-Yuryakh (1908)	40,000	1	0	12
Shandrin (1971)	42,000	2	1	13
Khatanga (1977)	> 50,000	6	2	14

Years in parentheses are the year of discovery of the samples. Total, number of samples available; Positive, number of samples from which amplifiable DNA could be extracted.

and that samples of a moderate age (up to 100,000 years) should be investigated to establish if they contain retrievable DNA. We completely agree and believe that it represents a great danger to the field of molecular archaeology if methods and procedures that allow the confirmation of results are not used. For example, sequences retrieved by molecular cloning (see ref. 5) cannot be reproduced because of the low cloning efficiency of ancient DNA, and are therefore of only limited scientific value.

To show that it is possible to reproduce results from Pleistocene faunal remains, we have extracted⁶ DNA from soft tissues of five different mammoths which vary in age from 9,700 to more than 50,000 years old. From four of the animals, more than one sample was available (see table above). Enzymatic amplification of a 93-base-pair fragment of the mitochondrial 16S ribosomal RNA gene yielded an amplification product from four of the five individuals, specifically seven of the fifteen extracts. All amplification products were directly sequenced in both directions. Products stemming from the same

DNA from ancient mammoth bones

SIR — The recovery of DNA from ancient human and animal remains is a topic of considerable interest. Controversy still surrounds the analysis of DNA sequences from biological samples aged many millions of years, for example from Miocene plants in anoxic sediments or insects in amber¹. Lindahl has suggested that moderately ancient DNA (about 100,000 years old) should be targeted for analysis to bridge the temporal gap that exists between DNA sequences from relatively recent biological remains and those many millions of years old². We report here the successful polymerase chain reaction

(PCR) amplification and sequencing of a fragment of the mitochondrial DNA cytochrome *b* gene, amplified from bones of two Siberian woolly mammoths dated at least 47,000 years before present (BP). To our knowledge, these mammoth bones are the oldest dated vertebrate remains from which DNA has been amplified.

The woolly mammoth, *Mammuthus primigenius*, was widely distributed across northern Eurasia and North America in the late Pleistocene. *Mammuthus* is thought to have originated in Africa about 5 million years ago, where it shared a common ancestor with the two living

	10	20	30	40	50	60	70	80	90	
Yuribei	AAGAAAAAA	CCTCCGAACG	ATATTATAAT	TCAGACTTTA	CAAGTCAAGA	TTCACATAATC	GCTTATTGA-	CCCAATACTT	GATCAACGGA	ACA
Dima										
Shandrin	G.....			C.....	A.....		T.....	T.....		
Khatanga	G.....			C.....	A.....		T.....	T.....		
Indian elephant				C.....	A.....	T.....				
African elephant	T.....		T.....	C.....	A.....	T.....				
Cow	..T...T..	..G..	..T...A..GA	CT...CC-	...AT	CA..T..	...C...T	..A..A..	...	...
Horse	..C...C..	...GT.	..T...-A..	C...A..	..C...A..	..AT...-	A...T...T	..A...CCA..	...	...
			*****	**	**	*****				

Sequence alignment of four mammoths, African and Indian elephant, cow and horse. Dots, sequence identity; dashes, deletions; asterisks, positions that cannot be unambiguously aligned.

Lox	T	TTC	GGC	TCA	CTA	CTA	GGA	GCA	TGC	CTA	ATT	ACA	CAA	ATC	CTA	ACA	GGA	TTA	TTC	CTA	GCC	ATA	CAT	TAT
Mam1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Mam2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Ele	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

Lox	ACA	CCC	GAC	ACA	ATA	ACT	GCA	TTT	TCA	TCT	ATA	TCC	CAT	ATT	TGC	CGA	GAT	GTA	AAC	TAC	GGC	TGA	ATT
Mam1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Mam2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Ele	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

Lox	ATT	CGA	CAA	CTA	CAC	TCA	AAC	GGA	GCA	TCC	ATT	TTC	TTC	CTC	TGC	CTA	CAC	ATT	GGA	CGA	AAC	ATC	TAC
Mam1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Mam2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Ele	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

Lox	TAT	GGG	TCC	TAC	CTA	TAC	TCG	GAA	ACT	TGA	AAT	ACC	GGC	ATT	ATA	TTA	CTA	CTA	ATC	ACC	ATA	GCC	ACC
Mam1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Mam2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Ele	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

Alignment of DNA sequences obtained from the two Siberian mammoths (Mam 1 and 2) and one Asian elephant (Ele) with the published African cytochrome *b* sequence (Lox)⁸ (GenBank accession number X56285); identity to this sequence is denoted by a full stop (.). The sequence was read from bases 96 to 378 of the cytochrome *b* gene⁸.

species of elephantid, the African elephant *Loxodonta africana* and Asian elephant *Elephas maximus*³. Mammoth remains include not only bones and teeth from thousands of localities, but also whole carcasses frozen in Siberian and Alaskan permafrost.

We extracted DNA from bones of two frozen Siberian woolly mammoths, and subsampled both specimens for accelerator mass spectrometry (AMS) radiocarbon dating at the Institute of Geological and Nuclear Sciences, New Zealand. The first individual, the Khatanga mammoth, consisted of the partial carcass of an adult male excavated in 1977 from alluvial sand in the eastern Taimyr peninsula⁴. We cut a wedge of cortical bone from one humerus (no. 31829) at the Zoological Institute, St Petersburg. Previous conventional radiocarbon dating on a sample of trunk and skin yielded dates of $\geq 40,340$ years BP (LU-750) and $\geq 53,170$ years BP (LU-1057), respectively. Both these dates are consistent with the new bone AMS date of $>47,000$ years BP (NZA-3711). As the specimen is beyond the range of finite radiocarbon dating, the age of this mammoth must be $\geq 47,000$ years or older.

The second mammoth sample was from a mandible (no. PIN 3657-181) excavated in 1975 by AVS from the Allaikha river, northeast Siberia⁵. It was found in permafrost 16 m below the surface in section ANV-1, beneath a horizon radiocarbon dated to $>46,000$ years BP on autochthonous plant vegetation remains (GIN-1681). We removed a piece of bone for DNA analysis, and a subsample yielded an AMS date of $>47,000$ years BP (NZA-3712). The predominant fossils in the

fauna horizon were of the extinct lemming *Dicrostonyx simplicior*, an evolutionary precursor of the extant collared lemming *D. torquatus*. This suggests a late Middle Pleistocene age for the mammoth, not less than 150,000 years BP.

We extracted DNA from the two mammoth bones (0.7 g bone) as described previously⁶, in parallel with three forensic and three prehistoric human bone samples, and an extraction blank with no bone. DNA was amplified from the bone extracts using the PCR with the highly conserved primers L14841 and H15149, which define a 375-base-pair fragment of the cytochrome *b* gene⁷. All the bone extracts yielded phylogenetically meaningful DNA sequences. We performed amplification reactions in a laboratory where elephant DNA had not been handled before. Following amplification, we determined the DNA sequences of both strands and compared them with the previously published African elephant cytochrome *b* sequence⁸ (see figure above), as well as with the orthologous sequences of eight African and six Asian elephants (one of which is shown in the figure). Preliminary phylogenetic analysis of the sequences from all these individuals shows that the mammoths fall within the elephantid clade, with the close divergence times for the three genera reflected in a tight clustering of the *Mammuthus*, *Loxodonta* and *Elephas* sequences.

Previous studies using protein analysis have failed to resolve the trichotomy between the three genera. Using a radioimmunoassay, Lowenstein and colleagues found the albumins of *Mammuthus*, *Loxodonta* and *Elephas* to be 99% immunologically homologous, consistent with an evolutionary divergence between 3 and 5 million years ago⁹. Our results are unable to resolve this trichotomy conclusively, although parsimony and maximum likelihood analysis with dolphin and rhinoceros as outgroups suggest that *Mammuthus* and *Loxodonta* could be sister taxa in a monophyletic clade, in contrast to the anatomical evidence. Sequencing data from additional

informative mtDNA regions and a larger number of individuals, from both the living and extinct genera, are needed before the evolutionary relationships of the elephantids can be fully understood.

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Peptide design

SIR — Jameson *et al.*¹ reported the rational design of a modified cyclic peptide from the CDR3 region of CD4 receptors of T cells which displayed considerable therapeutic effects both *in vitro* and *in vivo* in the experimental mouse model for allergic encephalomyelitis. The significant *in vivo* biological activity of this reverse-sequence peptide probably results from the fact that it contains all D-amino acids.

Brady and Dodson² summarized, in an associated News and Views article, how such a peptide analogue with reversed peptide bonds can introduce stability to proteolysis and at the same time stereochemically preserve the topological surface of the side chains in the original L-amino-acid sequence.

Readers should be made aware that this general approach to modification of the peptide backbone has a rather rich history in the areas of peptide medicinal chemistry and bioorganic chemistry. Cyclic retro-inverso peptides³ and linear, partial retro-inverso pseudopeptides⁴ have long been used as relatively stable peptidomimetics for numerous bioactive peptides. A recent review by Chorev and Goodman⁵ provides entry into the literature of retro-inverso isomers in peptide design and synthesis.

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nm23-H1 mutation in neuroblastoma

SIR— Reduced expression of *nm23-H1*, which encodes the nucleoside diphosphate kinase A (ref. 1), is associated with a high potential for metastasis in some tumour types², but its expression is increased in aggressive neuroblastoma, a childhood tumour of variable outcome³. To investigate the role of *nm23-H1* in neuroblastoma, we looked for mutations in this gene in 24 primary neuroblastoma tumours at different stages of the disease using the polymerase chain reaction (PCR) combined with analysis by single-stranded conformation polymorphism (SSCP)⁴ and Southern blotting.

Three sets of primers covering the coding sequence of *nm23-H1* were used separately for PCR amplification of complementary DNA prepared by reverse transcription of tumour poly(A)⁺ RNA. Four tumours from patients with advanced disease (stages 3 and 4)⁵ gave an altered DNA conformation on SSCP analysis when the primers used for PCR encompassed nucleotides 211 to 502 (Fig. 1). All four tumours gave rise to the same band and a less intense neighbouring normal band.

We used cDNA from two of the four

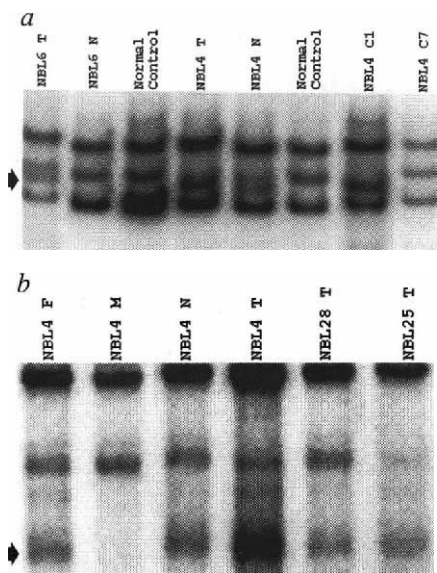


FIG. 1 PCR-SSCP analysis of *nm23-H1* cDNA prepared from tumour (T) and non-tumour (N) tissue from patients with neuroblastoma (NBL). cDNA was also prepared from peripheral blood lymphocytes obtained from normal control subjects and from the father (F) and mother (M) of NBL4. The variant band is indicated by an arrow. Patterns are shown for individual cDNA clones (C1 and C7) prepared from NBL4 tumour tissue. *a*, Samples were denatured and loaded onto a 6% polyacrylamide gel containing 10% glycerol, 90 mM Tris-borate, pH 8.3, and 4 mM EDTA⁴. Alternatively, in *b*, samples were run on a 0.5 X HydroLink mutation-detection enhancement gel.

tumours to generate cDNA clones which we analysed individually by SSCP: each clone gave either the variant band or the normal neighbouring band (Fig. 1*a*). When we sequenced the clones with altered conformation from these two tumours, we discovered an A-to-G nucleotide change in the coding region of *nm23-H1*, which resulted in a substitution by glycine for serine at position 120. This nucleotide swap creates a new cutting site for the restriction enzyme *HphI*, so the mutation can be readily detected.

From the four tumours giving an altered conformation by SSCP analysis, a new, smaller band (109 base pairs) was evident in *HphI*-digested cDNA which was not seen in cDNA from normal individuals; this band was the same size as the fragment resulting from the extra *HphI* restriction site created by the substitution (Fig. 2). When we screened cDNA from 64 normal individuals for the Ser 120 → Gly substitution using PCR-SSCP and *HphI* restriction analysis, we found normal patterns in all 64 samples.

Non-tumour tissue was available for two of the four patients whose tumour contained the Ser 120 → Gly substitution. One patient (NBL6) gave no abnormal bands from normal tissue (Fig. 1), indicating that the substitution was probably a result of a somatically acquired mutation; another (NBL4) had roughly equal amounts of normal and variant *nm23-H1* in cDNA from normal tissue (Fig. 1). Evidence for inheritance of a variant allele in patient NBL4 was obtained by SSCP analysis of cDNA from the parents. The father's, but not the mother's, cDNA had the same altered conformation as that from NBL4's normal tissue (Fig. 1*b*).

There was a small increase in *nm23-H1* gene copy number in 4 of the 24 primary tumours, based on phosphorimaging of Southern blots⁶. All four tumours were from patients with advanced disease. The highest gene copy number (10 copies) was found in NBL4's tumour with the inherited Ser 120 → Gly substitution. This tumour carried predominantly the form of *nm23-H1* with the substitution (Fig. 1). Southern blot analysis of the *nm23-H2* gene, which has been mapped to the same region (17q21) as *nm23-H1* (ref. 7), showed no evidence of increased *nm23-H2* gene copy number in any of the neuroblastoma tumours.

We confirmed that the substitution was a somatically acquired mutation in the case of NBL6, using two oligonucleotide primers for PCR amplification of a 123-base-pair genomic fragment spanning this substitution at nucleotide 442. *HphI* digestion of the PCR product from normal tissue showed that an A-to-G substitution was present at position 442, giving a

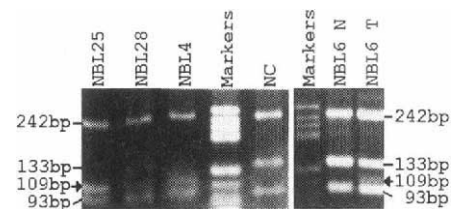


FIG. 2 *HphI* digestion of *nm23-H1* cDNA from the four tumours giving the same altered band by SSCP, and of non-tumour tissue (N) for NBL6 and a normal control (NC). The A-to-G substitution at position 442 creates an additional *HphI* digestion site in a 133-base-pair (bp) fragment which results in 109-bp (arrow) and 24-bp fragments. The 133-bp fragment is seen in the control and is faint or absent in NBL25, 28, 4 and 6.

75-base-pair fragment for NBL4 but no fragment for NBL6; the conclusion was the same when the 123-base-pair PCR-amplified genomic fragments were analysed by SSCP. This substitution must therefore be inherited in the case of NBL4 and a somatic mutation in the case of NBL6.

Genomic material was also analysed for an additional group of 26 neuroblastoma patients for whom paraffin-embedded tissue blocks were available⁸. DNA from two of 14 patients with advanced neuroblastoma showed altered bands attributable to the A-to-G substitution in their tumour, but not in normal tissue. None of 12 tumours from patients with limited-stage neuroblastoma showed evidence of this substitution. Genomic DNA from 36 normal individuals lacking this substitution in their cDNA, from 26 breast carcinomas and 17 acute leukaemias was also examined by *HphI* digestion of the amplified 123-base-pair fragment. No A-to-G substitution was detected in this group of 79 samples, excluding the possibility that this substitution represents a common polymorphism.

Our results show that a Ser 120 → Gly substitution is present in 6 of 28 advanced tumours but not in any of 22 limited-stage tumours, indicating that the mutation could be a feature of advanced neuroblastoma. We will show elsewhere that the mutant enzyme identified here still retains its catalytic activity but is more susceptible to denaturation, like the conditional lethal nucleoside diphosphate kinase mutation

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Killer of Prune in *Drosophila melanogaster*^{9,10}. The effects of the *nm23-H1* gene in tumour progression and metastasis probably depend on both its level of expression and the occurrence of specific mutations in its protein.

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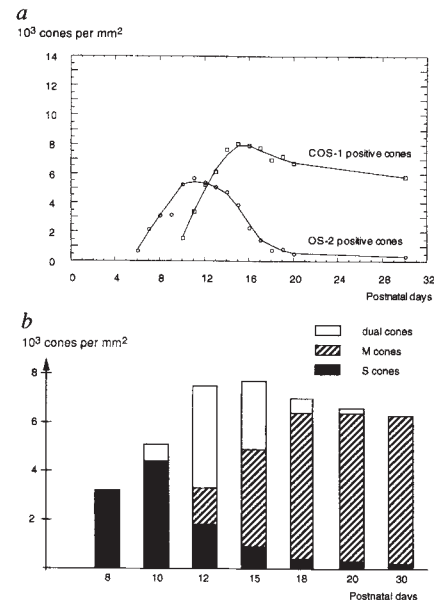
Retinal cone differentiation

SIR — During retinal development, differentiation of each cell type follows a strict timetable: in rodent retinae^{1,2} the short-wave-sensitive cone photoreceptor molecule (S pigment) is expressed before the middle-wave-sensitive (M) cone pigment, and we now show that this appearance of M cones after the S cones is a result of their development from S cones. So S-pigment expression must be the default pathway in cone differentiation, with prospective M cones switching to the green-sensitive pathway only secondarily in response to an as yet unknown factor.

We followed the kinetics of expression of S and M cones in the developing rat and gerbil retina with OS-2 and COS-1, antibodies specific for the carboxyl terminus of the S and M pigments, respectively³. In the rat, immunopositive S cones started to appear on the fifth postnatal day (P5) and their density increased until P11; the first COS-1-positive M cones emerged at P9, and reached the maximum density at P15 (see *a* in figure). The number of the OS-2-positive cones then dropped by almost 95 per cent, whereas COS-1-positive cones decreased only slightly.

This sudden drop in the S-cone fore-runners coinciding with the accumulation of M cones indicated that the S cones might be changing phenotype and transforming into M cones rather than dying out. Although programmed cell death probably influences adult patterns, we saw no degenerating or dying cones during the critical period of cone development, making this an unlikely explanation for the fall in S cones. Neither can it be accounted for by the roughly ten per cent expansion of the retinal surface between P10 and P30, nor by the lateral movement of cones during morphogenesis, as both cone types are uniformly distributed across the entire retina at all ages.

Owing to the rapid randomization of newly synthesized outer-segment proteins

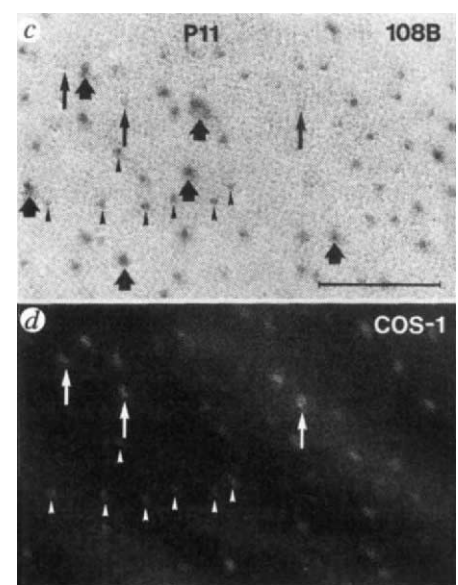


a, Quantitative analysis of cone development in rat retinae. OS-2- and COS-1-positive cones were counted on unit (0.1 mm²) areas and plotted as a function of postnatal time. The bound antibodies were detected with rhodamine and fluorescein isothiocyanate (TRITC and FITC, respectively). Topographically identical areas, located halfway between the optic nerve head and the superior edge of the ora serrata, were selected for comparison. Mean values derived from three animals of the same age were used. Cones expressing S pigment appear earlier (OS-2, circles), but after reaching their peak, the curve drops steeply. M-pigment-containing cones emerge later (COS-1, squares), and after peaking, the curve declines only slightly to plateau just below the maximum. *b*, Diagram showing the densities of cones stained with either 108B, a rabbit polyclonal antibody raised against a C-terminal epitope of the human blue visual pigment⁶ (S cones), or COS-1 (M cones), or both (dual cones). Bound antibodies were detected with FITC and TRITC, respectively, on double-labelled rat retinae. Note that dual cones arise during only a short period of the development. Samples shown are from identical areas of individual retinae, but the entire retina contained the same distribution of differently stained cones at each age. *c*, *d*, Double immunolabel of an 11-day-old rat retina with 108B and COS-1. Bound antibodies were detected by diaminobenzidine (108B) and FITC (COS-1). Pairs of photographs were taken from the same retinal area using Nomarski optics (*c*) and a fluorescent filter (*d*). Cones are mostly stained with both antibodies (arrowheads). There are a few heavily labelled 108B cones (genuine S cones) that are not recognized by COS-1 at all (thick arrows). Cones strongly labelled by COS-1 generally bind 108B only weakly (thin arrows). Scale bar, 25 μ m.

and the much slower shedding of the apical disks^{4,5}, cones containing both pigments should be evident during the proposed shift. We used double-label immunocytochemistry to test for such cones in the rat and gerbil, by reacting whole retinae with 108B, an antibody against human blue opsin⁶, and with the M-cone-specific COS-1; bound antibodies were detected using different fluorochromes or with diaminobenzidine and fluorochrome. We also reacted consecutive tangential sections alternately with OS-2 and COS-1 to identify with both pigments (not shown). OS-2- and 108B-labelled photoreceptors are known to be identical⁷.

Between P9 and P20, many cones bound both antibodies (*b* in figure), indicating the temporary coexistence of both pigments. Initially, all cones were recognized exclusively by S-pigment-specific antibodies. Later, the majority of cones were seen to be at various stages of transition between the two phenotypes (*c* and *d*). By P30 most of these cones were undetected by 108B and OS-2 and only a few, genuine S cones retained the original phenotype.

Our results show that most of the early-maturing S cones in the rat and gerbil



retina change their phenotype to become M cones. The green-sensitive cones thus derive from the transforming cones, while a few cones do not undergo the shift and constitute the definitive S-cone population. The differentiation around the second postnatal week indicates that instead of colour-specificities being predetermined, the ultimate cone phenotypes will be defined only after the onset of visual pigment synthesis.

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A cultured tour of the ineffable

Brian Aldiss

Aldous Huxley, born a hundred years ago last week, was a man of science, as well as a prophet, mystic, wit and satirist. A reappraisal of his writings reminds us of the immense diversity and enduring influence of his ideas.

"THE ambition of the literary artist is to speak about the ineffable, to communicate in words what words were never intended to convey. . . . In spite of 'all the pens that ever poets held' — yes, and in spite of all the scientists' electron microscopes, cyclotrons, and computers — the rest is silence, the rest is always silence." Thus Aldous Huxley, in a small book entitled *Literature and Science*, published in the year he died, 1963. He was still busy making connections, and in this case entering the debate between Snow and Leavis on "The Two Cultures". As ever, Huxley builds bridges. "The precondition," he tells us, "of any fruitful relationship between literature and science is knowledge."

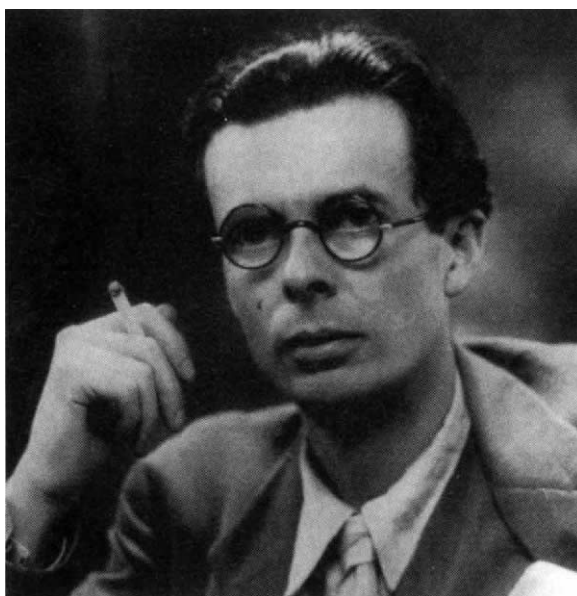
Which is all very well, but few of us set out in life with such cultural advantages as Huxley. He was the grandson on his father's side of the great Thomas Henry Huxley, while his mother was the grand-daughter of Dr Arnold of Rugby, niece of Matthew Arnold, and sister of Mrs Humphry Ward, the novelist. Aldous grew up in a house full of books and the intelligent conversations that spring from (and into) books, where knowledge is as routine as good meals.

This privileged English existence, with its Eton and Balliol background, was to end on the coast of California, fading out in gurudom, on an LSD injection. It is an extraordinary trajectory of a twentieth-century life, prompted in part by that fearless curiosity which Christopher Isherwood called "one of Aldous's noblest characteristics".

Curiosity spells diversity. The diversity of Huxley's life is echoed in his writings. These engage readers of many kinds, since even the novels, for which he was most renowned, touch upon religion, science, politics, literature, art, music, psychology, Utopianism and — to use the title of one of his most remarkable books — the human situation. Through them all flow Huxley's wit and his adroit use of language. His early distaste for bodily functions, in his Bright Young Thing phase, develops into a passion for mysticism. Henry Wimbush's comic discourse on sixteenth-century privies in *Crome Yellow* (1921) ("the necessities of nature are so base and brutish that in obeying them we

are apt to forget that we are the noblest creatures of the universe") becomes a preoccupation with enlightenment.

Thousands of individuals have had their spirit of enquiry sharpened by Huxley's cultured tour of "the ineffable". His preoccupation took many forms — advice on how to see, for instance, in his valuable little book *The Art of Seeing* (1942), in which one notes a typical observation



Aldous Huxley, from the cover of *The Hidden Huxley: Contempt and Compassion for the Masses* edited by David Bradshaw, a collection of various articles and broadcasts from 1920 to 1936. Faber and Faber, £17.50.

concerning the eye: "its peculiarly intimate relationship with the psyche". For Huxley, all things connected.

Concern with quality of life, and with any new thing that might conceivably alleviate the human condition, from hydroponics to Scientology, paved a way to the future. So — discounting the marvelous cruel joke of *After Many a Summer* (1939), with its speculations on evolutionary longevity — Huxley's three Utopian novels appear. None was received with great understanding by a literary world partial to country houses, large doses of characterization and even larger doses of nostalgia.

The trilogy consists of *Ape and Essence*, reviled on its appearance in 1946, perhaps because Huxley does not treat the human species with the respect it believes it deserves; *Island* (1963), his last novel; and *Brave New World* (1932), his most famous

work. Huxley was a connoisseur, some might say victim, of unconventional ideas. *Brave New World* shows us what happens when mass production is applied to biology. Promiscuous sex helps to preserve immaturity; immaturity reinforces superficiality. "When the individual feels, the community reels." Drugs keep everyone happy. The workers get four half-gram tablets of *somma* every day after work. It can readily be seen why thousands have longed to live in such a Utopia, from which Huxley himself would have recoiled, at least in part.

In *Ape and Essence*, the theme of sexual promiscuity is reintroduced. Nuclear bombs have fallen on Los Angeles. What remains of mankind has reverted to savagery. Womankind has reverted, apeline, to oestrus. For religion, a kind of perverted bogomilism is practised. The Narrator (the novel takes the form of a film script) says, "Thanks to the supreme Triumph of Modern Science, sex has become seasonal, romance has been swallowed up by the oestrus, and the female's chemical compulsion to mate has abolished courtship, chivalry, tenderness, love itself".

The codified society of *Brave New World* was the work of Mr Mond. In *Ape*, the ruined postwar world is the work of Belial. It's an emetic work, perhaps, but I persist in seeing both books as slyly sardonic, like *Gulliver's Travels*. If they are science fiction, they are Huxley's brand, not H. G. Wells's.

Huxley plays Jung to Wells's Freud. Huxley saw an escape from the human situation through mysticism, not politics as Wells did. So the least (but lengthiest) of his three Utopias, *Island*, reverses many of the assumptions of *Brave New World*, to show a threatened Utopia where *somma* and sexual freedom bring about genuine happiness. Alas, as Milton's *Paradise Regained* is less readable than its predecessor, *Paradise Lost*, so *Island* is less readable than its two predecessors. The devil — and Huxley knew it well — has all the best tunes. Mysticism and soil conservation challenge any novelist's repertoire.

Whereas Wells believed that a group of good men and true could reorganize the whole world, Huxley mistrusted govern-

ments. "Society," he said, "can never be greatly improved, until such time as most of its members choose to become theocentric saints."

This remark comes in *Grey Eminence* (1941), one of his most absorbing books, where his gifts are deployed in a study of Father Joseph, a Capuchin monk who became adviser to Cardinal Richelieu. Between them, Father Joseph and Richelieu prolonged the Thirty Years War, causing millions of deaths by torture, famine, disease and the usual appurtenances of war. Politics betrays the nationalistic religion, and vice versa.

The best of Huxley is scattered everywhere, perhaps most thickly in collections of essays; of the essay form he is one of the century's masters. "Adonis and the Alphabet" (1956) is a perfect example. The erudition, never obtrusive, carries us from psycho-industrial power, dirt and spirituality, population pressures, to Martian language and literature, and much else besides.

The Human Situation (1978) collects lectures delivered at Santa Barbara in 1959. They provide witty, learned summaries of Huxley's thinking on many subjects, answering such questions as:

"How should we be related to the planet on which we live? How are we to develop our industrial potentialities?" No better handbook to our ongoing civilization could be devised.

Huxley's continuing influence was summed up by Isaiah Berlin: "He was the herald of what will surely be one of the great advances in this and following centuries — the creation of new psychophysical sciences, of discoveries in the realm of what at present, for want of a better term, we call the relations between body and mind."

Almost everything Aldous Huxley wrote was adversely criticized at one time or another. Everyone spoke well of the man himself. His gentleness, sweetness and humour were remarked on by all those fortunate enough to know him. □

Brian Aldiss is a novelist and critic who lives in England. His latest novel, Somewhere East of Life, was published by HarperCollins on 1 August.

■ All of Huxley's works (47 books) are available in the United Kingdom from Flamingo, a paperback imprint of HarperCollins.

Discerning relationships

John F. Y. Brookfield

Molecular Markers, Natural History and Evolution. By John C. Avise. *Chapman and Hall*: 1994. Pp. 511. £75, \$89.95 (hbk); £24.99, \$37.50 (pbk).

DNA and proteins provide the most accurate guides to the phylogenies of living organisms. They allow the estimation of relationships between species, and between populations and individuals within species. They fail only when the concept of phylogeny itself fails, such as in dichotomies between trees of genes and species. In this important new book, Avise describes the uses of molecular techniques in answering some of the central questions of the population biology of diverse organisms. The first part deals with the methodology of various types of protein and DNA assays, and the ways in which either qualitative or quantitative data on genetic divergence can be used in phylogeny estimation. The second discusses the kinds of questions that molecular studies of variability have been able to answer. Examples range, with increasing genetic divergence, from studies of clonality, through kinship and speciation, to estimation of interspecific phylogenies. The closing chapter addresses the use of molecular tools in conservation genetics. The examples, many derived from the author's research team, are diverse and

unfailing interesting. The book is accurate and well written, and fulfils an important need as an undergraduate text.

I have misgivings about the field of molecular natural history itself, however. Pure biological research has simultaneously followed two distinct traditions. One of them involves the exhaustive cataloguing of organisms, their natural history and relationships. The other tradition, usually adopted by evolutionary and other geneticists, asks profound questions about model biological systems to discover general principles. The molecular tools developed in the latter endeavour can also be applied to the other organisms, but this conjunction of breadth of questions with depth of techniques can create an experimental programme that involves doing all of the experiments on all of the organisms. This task is impractical and there is no good reason for doing parts of it. For example, phylogeny estimations based on morphology have usually turned out to be correct, so further molecular checking could have a low priority. The studies in the many references here emphasize that molecular natural history is still natural history; real inductive generalizations across taxa are still required. □

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Dynamism and youth

Tom Spencer

Oceanic Islands. By Patrick D. Nunn. *Blackwell*: 1994. Pp. 413. £90, \$99 (hbk); £29.99, \$41.95 (pbk).

JUST as huge areas of the Earth, such as the 165 million km² of the Pacific Ocean, are usually squeezed into the last page of an atlas, so island geology, geomorphology and, to a lesser extent, biogeography rarely take scientific centre stage. Yet islands have a strong intellectual pedigree (one thinks of Darwin and Daly and, more recently, Menard and McArthur and Wilson) and, through their variations in origin, evolution, size and position, they offer ways of calibrating and testing theories of ocean geophysics and ecological processes. The remarkable re-evaluation in plate-tectonic terms of ocean basins as units of great dynamism and youth rather than areas of enormous antiquity and stability requires a corresponding reassessment of why islands form where they do, how they evolve into characteristic landscapes and acquire particular assemblages of species, and when and why they disappear. Such studies can now take advantage of the great advances that have been made in the past three decades in ocean-floor geophysics, palaeoclimatology, palaeoceanography and biochemical biogeography.

Oceanic Islands makes a brave stab at this modern synthesis. Using a rather loose definition of 'oceanic' and primarily concentrating on islands of 50–5,000 km², Patrick Nunn proceeds from a discussion of island formation and evolution in a plate-tectonic context, through island tectonics, climates, landscapes, coral reefs and sea-level changes, to island biotas and their disruption by human activity. The spread of subject matter is remarkably diverse and the geographical range global, although not unsurprisingly the author's field experience of the islands of the southwest Pacific features prominently. On the textbook spectrum from the encyclopaedic to the slim thematic volume (exemplified in this field by H. W. Menard's *Islands* (Scientific American Library/W. H. Freeman, 1986)) the book sits at the specialized end, although often only in the case studies does the complexity of disentangling tectonic, eustatic and climatic factors become totally clear. Some of the tables throw together in a rather uncritical way all the information that is available on a particular subject — Late Holocene sea-level evidence for example — which leads to some mind-bending problems of interpretation and often simply illustrates the variations in

the quality of data before and after the 1980s.

The book is valuable as an entry point to a large and scattered literature, for identifying unsatisfactory — or missing — data from particular island settings and for conveying the same mixed sensations that assailed Darwin on St Jago, Cape Verde Islands: "The scene, as beheld through the hazy atmosphere of this climate, is one of great interest; if, indeed, a person, fresh from the sea, and who has just walked, for the first time, in a grove of cocoa-nut trees can be a judge of anything but his own happiness." □

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Astronomical histories

Desmond King-Hele

History of Astronomy and Cosmology. By John North. Fontana (UK)/Norton (US): 1994. Pp. 697. \$35 (hbk); £12.99, \$18.95 (pbk).

Early Astronomy. By Hugh Thurston. Springer: 1994. Pp. 268. DM86, £34, \$49.

Planets, Stars and Orbs. By Edward Grant. Cambridge University Press: 1994. Pp. 816. £45, \$69.95.

Of the making of books on the history of astronomy there is no end, it seems, and here are three more, all admirable. Why does astronomy outdo all other scientific subjects in the popularity of its history? One relevant factor is its links with ultimate questions of how the Universe began and came to be as it is. Religion has tended to evaporate in countries where Mammon is the chief deity, and many people like to sample cosmology instead, as shown by the huge sales of Stephen Hawking's *A Brief History of Time*. The astronomy of the past also attracts scholars, because it is a triumph of human intellectual enquiry, usually being the most sophisticated and complex scientific construct of its time, even if later seen to be wrong.

John North's history of astronomy and cosmology is the fourth in a series on the history of science from Fontana/Norton. His book is a major achievement that trembles on the brink of being a masterpiece, although there are a few too many flaws to justify the last superlative. In the first 300 pages of narrative, North sweeps through pre-Keplerian astronomy with impressive thoroughness and confidence. All of human astronomical life is here, in chapters on prehistory, ancient Egypt, Mesopotamia, the Greeks and Romans,



The San Andreas Fault, taken from the cover of *Earthquakes and Geological Discovery* by Bruce A. Bolt, a well-illustrated popular account of earthquake case histories and geophysical studies that gives a flavour of the broad scientific accomplishments of seismology. W. H. Freeman/Scientific American Library, \$32.95, £19.95.

China and Japan, pre-Columbian America, India and Persia, and Islam. North is well qualified to write this book: he has a lifetime of experience in the history of astronomy and a narrative style that is a pleasure to read. He does his best to overcome the perennial problem of explaining spherical geometry to the uninitiated, with many diagrams to help. But nonspecialists may still find it all rather baffling, and pass on to the easier chapters.

North moves rapidly across the seventeenth and eighteenth centuries, lingers a little in the nineteenth, and then offers a full assessment of twentieth-century astronomy, right up to the feats of the Hubble Space Telescope. This modern section is quite different from the rest of the book, because its success depends on the selection of subjects and on fitting them into an integrated picture. Selection implies value judgement, and not everyone will agree with all of North's choices of important themes. To me, however, his analysis seems persuasive and reasonably well balanced, though deficient in scepticism, especially over cosmology, where ideas are apt to blaze up and fade away. (Cosmologists have an imaginative drive akin to fiction writers, and sometimes the similarity extends to the products.)

The book ends with a 35-page bibliographical essay and a splendid 38-page index. But readers should note that accented vowels come after all others in the index. Thus R might be expected to end with 'Ryle, M'; but after that comes

'Régis, P. S.' and 'Rømer, O.' (actually misprinted as 'Rmer'). The only other feature that deserves a warning is the preliminary page on numbers and units: for angles and time, North introduces an unfriendly sexagesimal system that needs careful definition; and unfortunately the definitions are marred by misprints. Overall, though, as an up-to-date general history of astronomy at a reasonable price, the book can be warmly recommended.

Hugh Thurston's *Early Astronomy* covers the same ground as North's first 350 pages, and the corresponding chapters are similar in title and length. Mesopotamian, Greek and Mayan astronomy, for example, receive 40, 73 and 8 pages respectively in North's book, and 18, 68 and 9 pages in Thurston's. So the difference is not in the substance but in the presentation. North's book is a continuous narrative addressed to a wide spectrum of readers; Thurston's is more of a scientific textbook, with much technical detail and numbered references. North's is a fairly cheap paperback; Thurston's a fairly expensive hardback. Both books have an attractive typeface, but North is disadvantaged because his signs for opening and closing quotations are the same as for minutes of arc (and he therefore cannot use the sign for minutes).

Thurston begins with a chapter on fundamentals: he discusses the constellations, the spherical geometry of the Sun, Moon, planets and stars, and the instruments used by early astronomers, including a long and useful section on armillar-

ies. The next eight chapters celebrate achievements in particular geographical areas, as already mentioned. Thurston goes into considerable scholarly detail, giving the vernacular words and notations in each of the diverse languages involved. Another good feature is the wealth of illustrations: there are 139 in all, mostly diagrams (for example of Stonehenge and of epicycles) and photographs of early sites and instruments. The main text ends with a long chapter on the European Renaissance, with emphasis on Copernicus and Kepler. There are six appendices covering points of detail, and an index. The author succeeds in his aim of tracing the growth of early astronomy in a clear and scholarly way, with technical detail.

Thurston says little about the scholastic mediaeval period from about 1200 to about 1500, when scientific advances in astronomy were few. Edward Grant's book fills this gap in no uncertain manner — presumably by chance, because nobody could imagine one publisher cooperating with another.

Planets, Stars and Orbs is a massive if somewhat mistitled book about the mediaeval cosmos from 1200 to 1687. The author, a professor at Indiana University, spent 15 years on the book, which runs to 816 large and closely printed pages. He has produced a detailed and authoritative study that is unlikely to be surpassed for many years to come. He begins by discussing Pierre Duhem's ten volumes on *Le Système du Monde*, which runs from Plato to Copernicus but is in Grant's opinion too rambling and disorganized to be as useful as it ought to be: hence his book, which is well organized. Many scientists regard scholastic Aristotelianism as the most sterile form of dry-as-dust learning — regurgitations of Aristotle untouched by real life or experiment. Grant, however, believes that "those who are comfortable with the fantastic ideas that make up the fabric of modern cosmology should suffer little or no distress confronting the claims of mediaeval cosmology". The difference is that the mediaeval scholars had to accommodate the perceived operations of God rather than the equations of relativity.

To imply that Grant's book is easy reading would be absurd: but he does his best to keep it lively by the Socratic device of continually asking questions. The title of Chapter 4 is "Is the world eternal, without beginning or end?". "Was creation from nothing?" and "Is the world perfect?" are others. After the main text there are 400 questions often asked by scholastic authors, with lists of sources where answers are attempted. For example, 18 authors, from Michael Scot to Illuminatus Oddus, consider whether there are, or could be, more worlds. The 400 questions elicit 1,176 responses: Roger Bacon has 37, William of

Occam 7 and John Major 40.

Professor Grant ploughs through this morass of mediaeval opinion and argument with unfailing zest and admirable rationality. His long history of time long past should be of lasting value to everyone interested in either scholasticism or cosmology. The three books together offer a complete education in history of astronomy up to the seventeenth century. □

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Shattered dreams

Abraham Verghese

How We Die. By Sherwin B. Nuland. Knopf/Chatto and Windus: 1994. Pp. 278. \$24, £15.99 (hbk); £8.99 (pbk).

IN the past year, two books, both by physicians, have soared to the bestseller lists in several countries. Both reached this pinnacle by virtue of lucid prose, aided in large measure by an unprecedented peak in public fascination with issues of self. In *Listening to Prozac*, Peter Kramer deals brilliantly with the complex issue of personality and questions whether a commonly used antidepressant can alter the very essence of a person's character as well as lift depression. In *How We Die*, Sherwin Nuland deals with death, our own death in this modern era, and what we can expect as the final curtain falls. Nuland's book is, like its title, straightforward and is a quick read, although it is not quite as intellectually engaging as Kramer's book.

Nuland tells one everything one would like to know about death — or, at least, as much as any living person could possibly tell one. Unlike *Embraced by the Light* by Betty J. Eadie, another popular book about death (or near death), Nuland for the most part avoids the mystical and writes from a basis in fact rather than speculation. His view of death is coloured by his own personal experiences, which he shares generously with us: the author as a boy of 11 losing his mother; witnessing his Aunt Rose's decline in health and eventual death; as a young medical student left to care for a patient dying from a cardiac arrest; as surgeon and writer coping with many more deaths in the course of a long career; and finally observing his brother's death from cancer and thereby coming to terms with his own mortality.

Nuland's strategy is to examine in individual chapters several common causes of death that plague modern society: heart disease, ageing and Alzheimer's disease, murder, suicide, euthanasia, AIDS and cancer. Each section has a prefatory sec-

tion covering anatomy and pathophysiology that medical and scientific readers may choose to skim through but other readers may find helpful. Nuland is always informative, making many references to historical figures and to works of literature, although at times he tends to adopt the tone of the lecturer at the podium and

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Blake's *The Descent of Man into the Vale of Death* (1808).

also to be grandiloquent, as in: "The force of life fills out our tissues with its pulsing vibrancy and puffs them up with the pride of being alive". But this is a minor quibble. Nuland is an experienced academic teacher, and we soon settle dutifully at our desks and listen to what he has to say.

In the last two chapters, "Lessons and Learned" and "Epilogue", we come to the core of the book, with a message to take away. Nuland tell us that the dream we have of a serene deathbed scene of leave-taking will be attained by very few. Disease and circumstances will often conspire to make such an end impossible. He suggests that we need to abandon this classic image; that dying with dignity is often beyond our control, even perhaps an impossible goal. Instead, he writes, "the greatest dignity to be found in death is the dignity of the life that preceded it". □

Abraham Verghese is author of My Own Country: A Doctor's Story of a Town and Its People in the Age of AIDS (Simon and Schuster; published in the UK by Phoenix as Soundings), to be reviewed in these pages on a later date. He is at the Texas Tech University Health Sciences Center, 4800 Alberta Av., El Paso, Texas 79905, USA.

Mechanism of activation of the TGF- β receptor

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Transforming growth factor- β (TGF- β) signals by contacting two distantly related transmembrane serine/threonine kinases called receptors I and II. The role of these molecules in signalling has now been determined. TGF- β binds directly to receptor II, which is a constitutively active kinase. Bound TGF- β is then recognized by receptor I which is recruited into the complex and becomes phosphorylated by receptor II. Phosphorylation allows receptor I to propagate the signal to downstream substrates. This provides a mechanism by which a cytokine can generate the first step of a signalling cascade.

THE TGF- β superfamily is a large group of cytokines which are among the most versatile carriers of growth and differentiation signals¹⁻⁶. Members of this family participate in setting up the basic body plan during embryogenesis in mammals, frogs and flies; control formation of neural tube, limbs, cartilage, bone and sexual organs; suppress epithelial cell growth, promote wound repair, and influence important immune and endocrine functions¹⁻⁶. Alterations in the activity of these factors in humans have been implicated in fibrosis, immunosuppression, cancer and other disorders^{3,7}.

Progress towards elucidating the mode of action of these factors has been made with the identification of their receptors⁸⁻²¹. TGF- β and related factors bind to sets of two membrane proteins called receptor types I and II^{1,2}. Genetic evidence from cell mutants resistant to TGF- β action suggests that TGF- β binding to receptor I requires the presence of receptor II and, furthermore, that both receptors are required for signalling of any response^{1,4,18,20-26}. These properties appear to be shared by receptors for other TGF- β family members^{14,17,26,27}.

Molecular cloning of types I and II receptors for TGF- β and the related activins and bone morphogenetic proteins (BMPs) has shown that they are a family of proteins with a small extracellular region, a single transmembrane segment, and a cytoplasmic region with a serine/threonine kinase domain^{1,2}. The type I receptor kinase domains are more similar to each other than they are to the type II receptor kinase domains, with which they show less than 40% amino-acid sequence identity. The type I receptors share various other structural features not found in the type II receptors, including the spacing of cysteines in the extracellular region and a highly conserved serine- and glycine-rich segment adjacent to the amino-terminal boundary of the kinase domain²¹.

Studies with the cloned TGF- β and activin type I receptors have confirmed their inability to bind ligand in the absence of type II receptors^{16,21}, and the failure of the latter to signal in the absence of type I receptors^{23,26}. Another feature of this system is the ability of type II receptors to interact with different type I receptor isoforms. Thus, the TGF- β type II receptor, T β R-II, can interact with at least two type I receptors, called T β R-I (also known as ALK5 (ref. 18) or R4 (ref. 20) and TSR-I¹⁷, whereas the activin type II receptors ActR-II and ActR-IIB can interact with at least three distinct activin type I receptors^{17,19,28,29}. The specificity of the biological response to ligand in a given cell type

appears to be defined by the particular type I receptor engaged in the complex, thus providing a basis for the multifunctional nature of these cytokines²⁶.

The basis for the requirement of TGF- β and related factors to interact with two distinct transmembrane kinases in order to signal has remained unsolved. This requirement is in contrast to the well-characterized tyrosine kinase receptors which act as ligand-activated homodimeric kinases, or dimers of highly related kinases, which autophosphorylate on tyrosine residues forming sites for association with various signal-transducing proteins^{30,31}. The pathways initiated in this manner, as those initiated by G-protein-coupled receptors, often lead to cytoplasmic serine/threonine kinases that act in sequence, with one phosphorylating and activating the next³². Here we describe the early events triggered by TGF- β binding to its receptors, and demonstrate that receptor II is a constitutively active kinase which uses the ligand to recruit, phosphorylate and signal through receptor I, thus generating the first step of a TGF- β signalling pathway.

TGF- β receptor II is constitutively active

Because ligand-stimulated autophosphorylation of tyrosine kinase receptors initiates their signalling process, we asked if the same would occur with the TGF- β type II receptor which binds ligand on its own. We examined the phosphorylation state of T β R-II in Mv1Lu cells, a mink lung epithelial cell line whose TGF- β receptors and responses are well characterized^{33,34}. Human T β R-II constructs tagged at the carboxy terminus with an influenza virus haemagglutinin epitope (HA) were used. The functional properties of these and other tagged receptors used were similar to those of unmodified receptors, as determined by their ability to restore TGF- β binding and responsiveness when expressed in receptor-defective mutant Mv1Lu cells (refs 23, 26, and results not shown).

Immunoprecipitation of the transfected T β R-II from [³²P]phosphate-labelled cells with anti-HA antibody showed that this receptor was highly phosphorylated in the absence of TGF- β addition (Fig. 1a). Phosphorylation occurred on multiple serine residues, as determined by receptor phosphoamino-acid analysis and tryptic phosphopeptide mapping (Fig. 1b, c). Tryptic digestion of the receptor and analysis by two-dimensional thin layer electrophoresis and chromatography yielded eight phosphopeptides of similar labelling intensity and a ninth phosphopeptide (spot 1) which was more intensely labelled. The latter probably corresponds to the serine-rich C-terminal tail of T β R-II because it was missing in phosphopeptide maps (not

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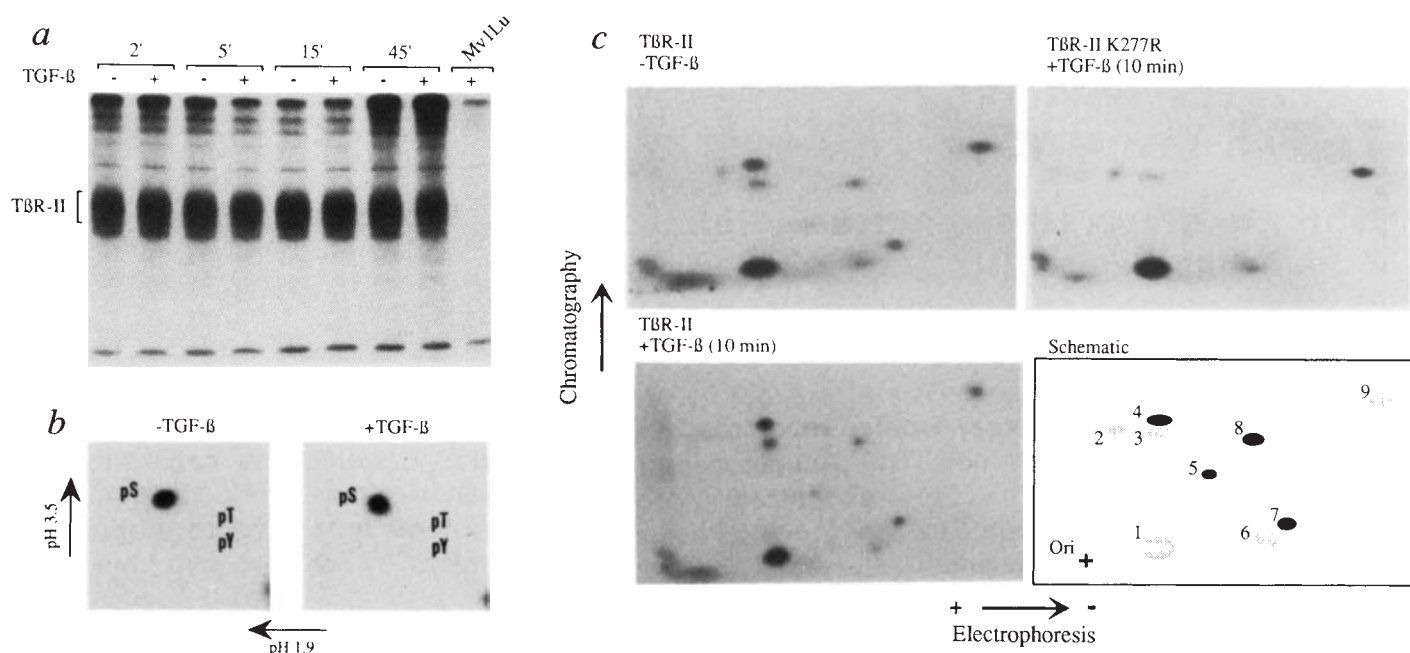


FIG. 1 **a**, Phosphorylation of T β R-II. Mv1Lu cells stably transfected with T β R-II tagged with an HA-epitope (T β R-II/HA; ref. 23) or parental Mv1Lu (Mv1Lu lane) were [32 P]phosphate-labelled and then incubated in the presence (+) or absence (-) of TGF- β for the indicated times. Cell lysates were immunoprecipitated with anti-HA antibody and analysed by SDS-PAGE and autoradiography. **b**, Phosphoamino-acid analysis of T β R-II. [32 P]phosphate-labelled T β R-II/HA transfectants were incubated in the presence (+) or absence of TGF- β , and T β R-II/HA was purified by immunoprecipitation, isolated by SDS-PAGE, and subjected to acid hydrolysis and phosphoamino-acid analysis⁴¹. pS, Phosphoserine; pT, phosphothreonine; pY, phosphotyrosine. **c**, Tryptic phosphopeptide mapping of T β R-II. R-1B cells transiently transfected with T β R-II/HA or kinase-defective T β R-II/HA(K277R) cDNAs in the pCMV5 expression vector^{23,26}, were [32 P]phosphate-labelled in the presence or absence of TGF- β and isolated by immunoprecipitation. Tryptic digests of gel-

purified T β R-II were resolved in two dimensions with electrophoresis in pH 1.9 buffer and chromatography in phosphochromatography buffer⁴¹. A schematic drawing of the tryptic phosphopeptides is shown with autophosphorylated peptides (black spots) and location of sample application (Ori+) indicated.

METHODS. Transiently transfected L17 cells, a highly transfectable clone of R1-B cells^{23,26}, were incubated in phosphate-free minimal essential media containing 1 mCi ml⁻¹ [32 P]phosphate for 2 h followed by 10 min with or without 1 nM TGF- β , washed once in ice-cold PBS and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X-100, 50 mM NaF, 10 mM sodium-pyrophosphate and 1 mM sodium-orthovanadate). Receptors were immunoprecipitated with anti-HA monoclonal antibody (12CA5; BAbCo)²³. Phosphoamino-acid analysis and tryptic-phosphopeptide mapping were done using the HTLE-7000 system (CBS Scientific Co.)⁴¹.

shown) of a mutant receptor, T β R-II(Δ tail), that lacks this tail yet has undiminished signalling activity³⁵.

Exposure of cells to TGF- β 1 (the TGF- β isoform used throughout these studies) for 2–45 min did not alter the overall phosphorylation level of T β R-II or the pattern of tryptic phosphopeptides (Fig. 1a–c). However, mutation of lysine 277 to arginine in the putative ATP binding site of T β R-II, which destroys the kinase and signalling activities of this receptor^{23,25}, caused loss of receptor phosphorylation. Specifically, four phosphopeptides (spots 4, 5, 7 and 8) that were present in tryptic digests of wild-type T β R-II were missing in the mutant receptor (Fig. 1c). The high level of expression of T β R-II in these cell lines precluded analysis of phosphorylation events that may occur because of complexing between T β R-II and the endogenous type I receptor. Nevertheless, these results suggest that T β R-II, in the absence of T β R-I, is constitutively phosphorylated by cellular kinases at multiple sites and by itself at additional sites, and that its autophosphorylating activity is not regulated by ligand binding.

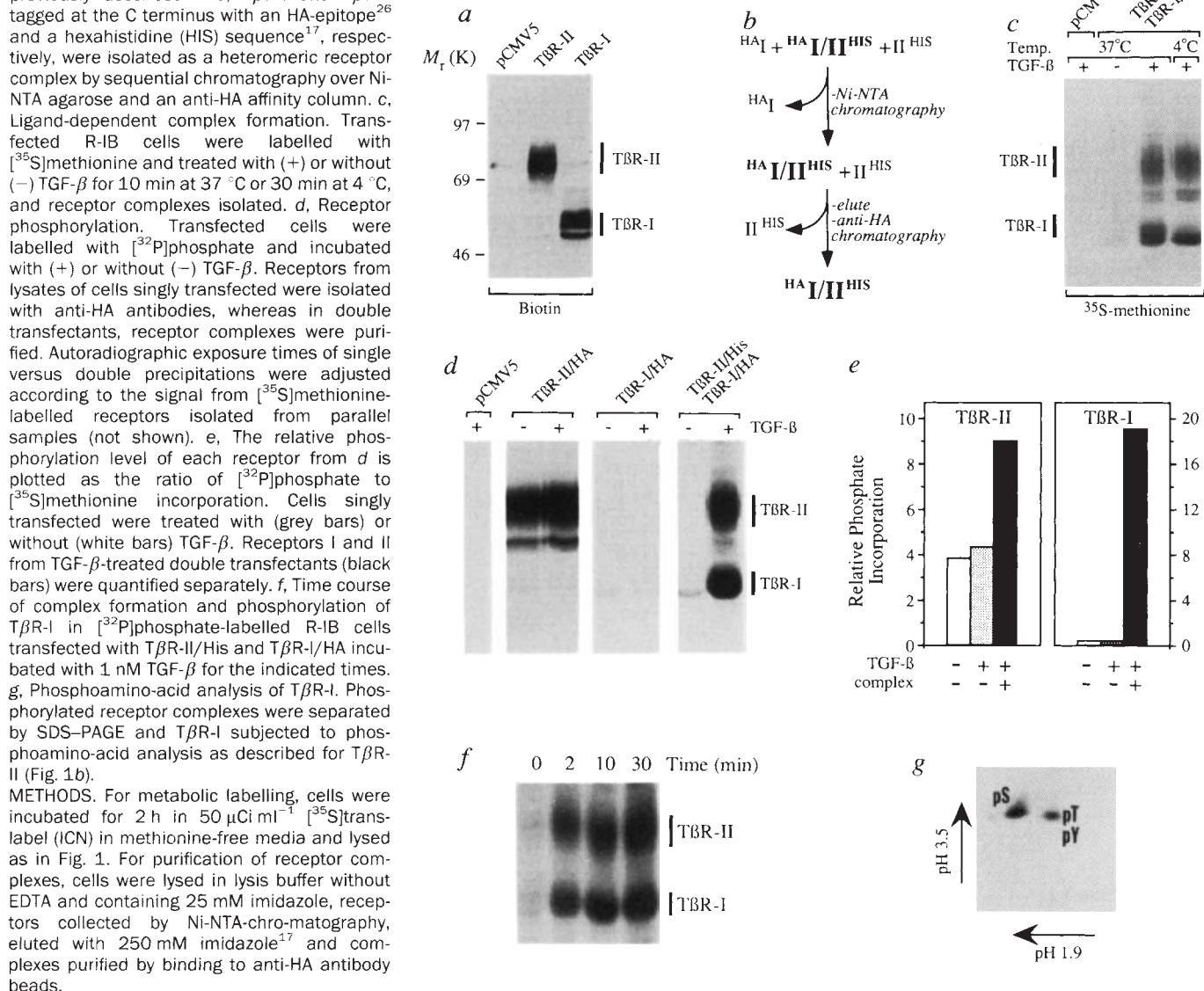
TGF- β recruits receptor I

To investigate why TGF- β type I receptors only bind ligand in the presence of T β R-II, we focused on T β R-I which with T β R-II signals cell-cycle arrest²⁶ and expression of various extracellular matrix proteins^{18,26}. Although T β R-I does not bind TGF- β when expressed alone, biotinylation of intact COS-1 cells transfected with T β R-I showed that this receptor was synthesized and reached the cell surface as efficiently as T β R-II (Fig. 2a). Therefore, the inability of T β R-I to bind ligand when expressed

in the absence of T β R-II was not due to a failure of T β R-I to reach the cell surface but to its inability to recognize free TGF- β .

When expressed together, T β R-I and T β R-II form a ternary complex with TGF- β ²³. To determine whether this complex pre-exists or is induced by the ligand, complementary DNA vectors encoding HA-tagged T β R-I (T β R-I/HA) and hexahistidine-tagged T β R-II (T β R-II/His) were expressed at high levels by transient transfection into the mutant Mv1Lu cell clone R-1B which is defective in T β R-I^{26,36}. Both receptors are expressed at roughly equal levels which are much higher than the endogenous TGF- β receptors, thus allowing for the analysis of transfected receptor complexes with minimal endogenous receptor background²⁶. To measure complex formation between transiently expressed T β R-I/HA and T β R-II/His, proteins were isolated under non-denaturing conditions from lysates of metabolically labelled cells through a two-step procedure using Ni-NTA-agarose that binds polyhistidine sequences followed by immunoprecipitation with anti-HA antibody and protein A-Sepharose (Fig. 2b). The results demonstrate that the association of T β R-I with T β R-II was highly dependent on ligand and occurred at physiological temperatures or in the cold within minutes of TGF- β addition to the cells (Fig. 2c). This complex was highly stable and could not be dissociated by exposure of purified complex to 4 M urea, 0.5 M LiCl, 1% SDS at room temperature, or pH as low as 2.5 but was disrupted by boiling in 1% SDS (data not shown). Receptors I and II transiently expressed at high level in these cells consistently displayed a trace amount of complex in the absence of ligand (Fig. 2c, lane 2), suggesting that these receptors may have a low intrinsic affinity

FIG. 2 *a*, Cell-surface expression of $T\beta R$ -II and $T\beta R$ -I in transiently transfected COS-1 cells was detected by cell-surface labelling with biotin as previously described⁴². *b*, $T\beta R$ -I and $T\beta R$ -II tagged at the C terminus with an HA-epitope²⁶ and a hexahistidine (HIS) sequence¹⁷, respectively, were isolated as a heteromeric receptor complex by sequential chromatography over Ni-NTA agarose and an anti-HA affinity column. *c*, Ligand-dependent complex formation. Transfected R-IB cells were labelled with [³⁵S]methionine and treated with (+) or without (-) TGF- β for 10 min at 37 °C or 30 min at 4 °C, and receptor complexes isolated. *d*, Receptor phosphorylation. Transfected cells were labelled with [³²P]phosphate and incubated with (+) or without (-) TGF- β . Receptors from lysates of cells singly transfected were isolated with anti-HA antibodies, whereas in double transfectants, receptor complexes were purified. Autoradiographic exposure times of single versus double precipitations were adjusted according to the signal from [³⁵S]methionine-labelled receptors isolated from parallel samples (not shown). *e*, The relative phosphorylation level of each receptor from *d* is plotted as the ratio of [³²P]phosphate to [³⁵S]methionine incorporation. Cells singly transfected were treated with (grey bars) or without (white bars) TGF- β . Receptors I and II from TGF- β -treated double transfectants (black bars) were quantified separately. *f*, Time course of complex formation and phosphorylation of $T\beta R$ -I in [³²P]phosphate-labelled R-IB cells transfected with $T\beta R$ -II/His and $T\beta R$ -I/HA incubated with 1 nM TGF- β for the indicated times. *g*, Phosphoamino-acid analysis of $T\beta R$ -I. Phosphorylated receptor complexes were separated by SDS-PAGE and $T\beta R$ -I subjected to phosphoamino-acid analysis as described for $T\beta R$ -II (Fig. 1*b*).



for each other. $T\beta R$ -II binds ligand independently of $T\beta R$ -I, suggesting that $T\beta R$ -I recognizes TGF- β bound to $T\beta R$ -II but not free in the medium or bound to other membrane proteins. Therefore, one function of the ligand is to help recruit $T\beta R$ -I into a stable complex with $T\beta R$ -II.

Receptor I phosphorylation

$T\beta R$ -I isolated as a complex with $T\beta R$ -II showed a heterogeneous electrophoretic migration pattern common in phosphorylated proteins: $T\beta R$ -I from complexes formed by incubation of cells with TGF- β 1 at 4 °C migrated as a single band on sodium dodecyl sulphate electrophoresis gels whereas $T\beta R$ -I from receptor complexes formed at 37 °C consistently migrated with slower mobility (Fig. 2*c*).

Analysis of receptors isolated from [³²P]phosphate-labelled cells confirmed that $T\beta R$ -I was phosphorylated when it was in a complex with $T\beta R$ -II. $T\beta R$ -II transfected alone showed a high level of phosphorylation, but this level was not increased on incubation of cells with TGF- β (Fig. 2*d, e*). Similarly, phosphorylation of $T\beta R$ -I expressed alone did not increase on TGF- β addition to the medium, although the basal level of phosphorylation was very low (Fig. 2*d, e*). However, when $T\beta R$ -I

was isolated as part of a ligand-induced complex with $T\beta R$ -II from cells cotransfected with both receptors, phosphorylation was almost 50-fold higher than when it was expressed alone (Fig. 2*d, e*). Ligand-induced phosphorylation of $T\beta R$ -I in receptor complexes was near-maximal 2 min after TGF- β addition to the cells (Fig. 2*f*), and occurred on serine and threonine residues at a ratio of about 3:1 (Fig. 2*g*). In contrast, the phosphorylation level of $T\beta R$ -II in ligand-induced complexes was increased less than twofold compared with $T\beta R$ -II expressed alone (Fig. 2*d, e*). This increase was quantitative because no new phosphopeptides were observed (see below). This modest increase could represent protection of phosphates against phosphatase activity in the complex or may represent differences in the [³²P]phosphate/[³⁵S]methionine ratios of the cell-surface receptor pool (isolated in the complex) versus the entire receptor pool (isolated in the anti-HA immunoprecipitate).

Receptor II phosphorylates receptor I

To determine whether ligand-induced phosphorylation of $T\beta R$ -I was catalysed by its own kinase activity or by the kinase activity of $T\beta R$ -II, we compared the phosphorylation state of these receptors when cotransfected with wild-type or kinase-defective

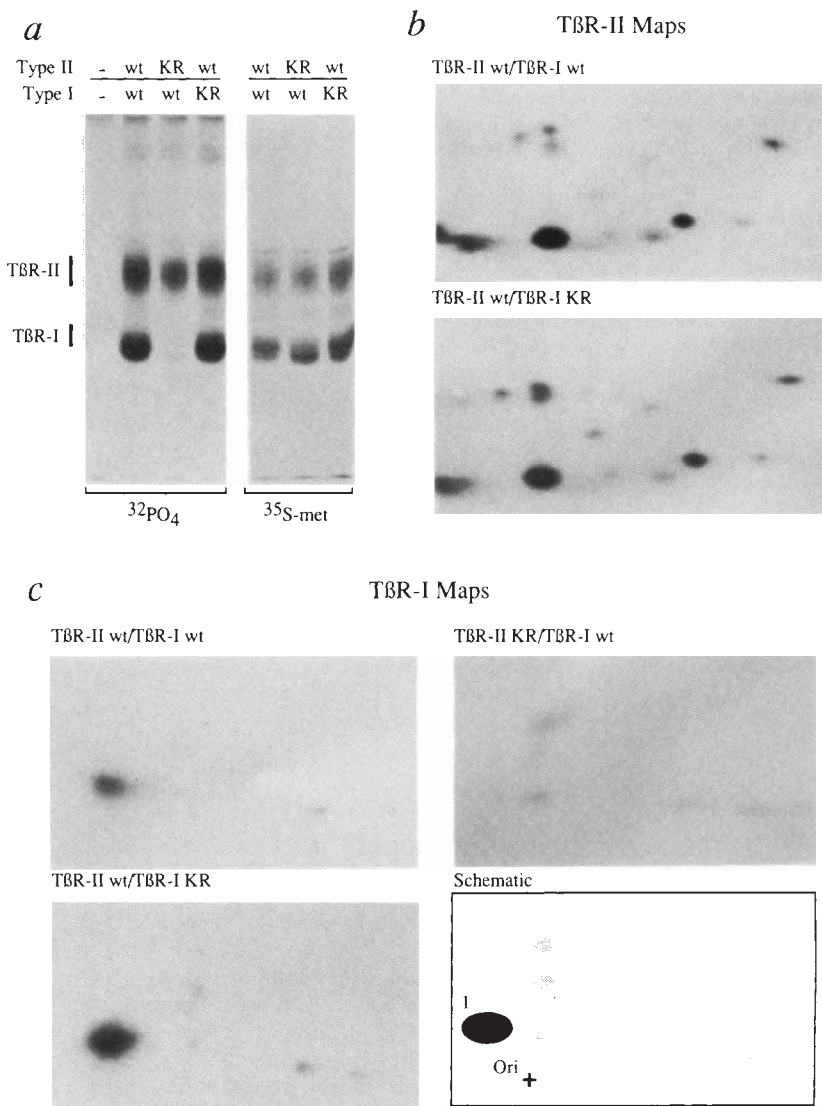
mutants of each other. When T β R-I was coexpressed with the kinase-defective receptor T β R-II(K277R), it bound ligand and formed a complex with this mutant receptor as efficiently as it did with wild-type T β R-II, but did not become phosphorylated (Fig. 3a). Mutation of lysine 232 to arginine in the putative ATP binding site of T β R-I destroys the kinase and signalling activities of this receptor^{20,26}. When this construct, T β R-I(K232R), was coexpressed with T β R-II, it bound ligand, formed a complex and became phosphorylated in a manner similar to wild-type T β R-I (Fig. 3a). These results suggest that the kinase activity of T β R-II is required for T β R-I phosphorylation.

This conclusion was supported by the results of receptor phosphopeptide mapping. Tryptic digestion of [³²P]phosphate-labelled T β R-I derived from ligand-induced receptor complexes yielded one major phosphopeptide and five minor ones (Fig. 3c). The major phosphopeptide contained phosphoserine and phosphothreonine in a 3:1 ratio as determined by phosphoamino-acid analysis (data not shown). This phosphopeptide was absent from T β R-I derived from complexes formed with T β R-II(K277R) (Fig. 3c). Furthermore, tryptic digestion of T β R-I(K232R) yielded a phosphopeptide map identical to that of T β R-I (Fig. 3c), indicating that T β R-I is not an autophosphorylating kinase in intact cells even when it participates in a receptor complex. Thus, ligand-induced phosphorylation of T β R-I within the receptor complex was dependent on the kinase activity of T β R-II.

Analysis of the tryptic phosphopeptide map of T β R-II isolated from complexes with T β R-I showed that it was identical to that of T β R-II in complex with T β R-I(K232R) (Fig. 3b). Furthermore, the pattern of tryptic phosphopeptide maps obtained from T β R-II in the complex and T β R-II isolated in the absence of T β R-I were the same (compare Figs 1b and 3b). Together these data indicate that T β R-II is not a substrate of T β R-I and that its phosphorylation is not qualitatively altered as a consequence of ligand-induced complex formation. Because the kinase activity of T β R-I is essential for signalling, but neither autophosphorylates nor phosphorylates T β R-II, these results collectively suggest that ligand-bound T β R-II recruits and phosphorylates T β R-I which then phosphorylates downstream substrates.

To obtain direct evidence that T β R-I is phosphorylated by the kinase activity of T β R-II, we did *in vitro* kinase assays using ligand-induced complexes formed in intact cells. To generate receptor complexes containing a minimal level of phosphorylation, cells were incubated with TGF- β in the cold. The receptors were purified from cell lysates using Ni-NTA-agarose beads and phosphorylation was allowed to proceed in the presence of [γ -³²P]ATP. Phosphorylated receptors were eluted by boiling in SDS and type I receptors collected by immunoprecipitation with anti-HA antibodies. Phosphorylation of T β R-I was observed when coexpressed with wild-type T β R-II receptor but not the kinase-defective version, T β R-II(K277R). Both wild-type and

FIG. 3 a, Phosphorylation of T β R-II and T β R-I in complexes formed between wild-type and kinase-deficient receptors. R-1B cells were transiently transfected with the indicated combinations of wild-type (wt) T β R-II/His and T β R-I/HA or their kinase-deficient (KR) derivatives T β R-II/His(K277R) and T β R-I/HA(K232R). Cells were labelled with either [³²P]phosphate or [³⁵S]methionine, and incubated with TGF- β for 10 min at 37 °C. Receptor complexes were then purified, resolved by SDS-PAGE and receptors visualized by autoradiography or fluorography. The similar methionine content in receptor II and receptor I allows direct comparison of labelling intensities to assess the amounts of receptor proteins. b, c, Tryptic phosphopeptide mapping of T β R-II and T β R-I isolated from ligand-dependent complexes. [³²P]phosphate-labelled complexes between the indicated receptor combinations were generated as described and shown in a. Tryptic phosphopeptides from T β R-II (b) or T β R-I (c) were resolved in two dimensions⁴¹. A schematic diagram of the phosphotryptic peptides for T β R-I is shown with the T β R-II kinase-dependent peptide (spot 1) and the location of sample application (Ori +) indicated.



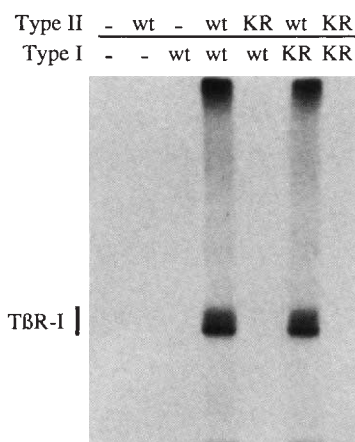


FIG. 4 Phosphorylation of T β R-I by T β R-II *in vitro*. R-1B cells were transiently transfected with wild-type (wt) T β R-II/His and T β R-I/HA or their kinase-deficient (KR) derivatives T β R-II/His(K277R) and T β R-I/HA(K232R), either singly or in combination, as indicated. Cells were incubated with 1 nM TGF- β for 15 min at 4 °C, lysed and receptors collected by binding to Ni-NTA-agarose. These samples were incubated in kinase assay buffer containing 50 μ M [γ - 32 P]ATP²⁶. To separate receptor I from receptor II and unincorporated [γ - 32 P]ATP, complexes were eluted from Ni-NTA-agarose by boiling in 1% SDS, diluted 10-fold in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X-100, 1% Na deoxycholate (see Fig. 5) and T β R-I isolated by immunoprecipitation with anti-HA antibody.

kinase-defective T β R-I(K232R) were similarly phosphorylated (Fig. 4). Tryptic phosphopeptide maps of T β R-I phosphorylated *in vitro* were similar to those of T β R-I phosphorylated in intact cells except that the ratio of incorporated phosphate varied, with the relative level of spot 1 being lower than in T β R-I phosphorylated in intact cells (data not shown). Thus receptor I in the TGF- β receptor complex is directly phosphorylated by receptor II.

Receptor complexes [32 P]-labelled *in vivo*

To identify the regions of T β R-I phosphorylated by T β R-II, [32 P]-labelled receptor complexes *in vivo* were purified by Ni-NTA chromatography, eluted by boiling in SDS to separate the two receptors, and T β R-I was collected by immunoprecipitation. Purified T β R-I was subjected to digestion with chymotrypsin and V8 protease with each protease generating a single phosphopeptide fragment with an approximate relative molecular mass (M_r) of 3,500 and 2,800 (3.5K and 2.8K), respectively (Fig. 5b). Analysis of the sequence for T β R-I revealed that only the GS domain, a region highly conserved in type I receptors from *Drosophila* through to humans^{1,21,29} could yield both chymotryptic and V8 fragments with these mobilities (Fig. 5a). To determine the effect of phosphorylation on receptor I function, we constructed a mutant form of T β R-I(T β R-I(TS185-204VA)) in which the threonine and serine residues encompassed by the V8 digestion sites were mutated to valine and alanine, respectively. T β R-I(TS185-204VA) was essentially equal to T β R-I in its ability to be expressed, bind TGF- β with T β R-II and form a complex with this receptor (data not shown). However, analysis of receptor complexes purified from [32 P]phosphate-labelled cells showed that T β R-I(TS185-204VA) was unable to undergo ligand-induced phosphorylation (Fig. 5c). Together with the chymotryptic and V8 protease maps these results suggest that the GS domain is the site of phosphorylation by T β R-II.

To test the signalling capacity of T β R-I(TS185-204VA), we used the TGF- β resistant R-1B cells which lack functional TGF- β type I receptors. Cotransfection of T β R-I and a luciferase reporter gene under the control of the TGF- β -responsive promoter 3TP²³ restored the responsiveness of these cells to

TGF- β (Fig. 5d) as previously described^{20,26}. In contrast, cotransfection of the reporter with T β R-I(TS185-204VA) failed to restore TGF- β -inducible gene expression (Fig. 5d). Similar results were obtained when two other TGF- β responses, elevation of plasminogen activator inhibitor-I and fibronectin expression²⁶, were analysed (data not shown). To assess the antiproliferative signalling capacity of these receptors, R-1B cells stably expressing either T β R-I or T β R-I(TS185-204VA) were generated. T β R-I restored the full antiproliferative responsiveness of R-1B cells whereas T β R-I(TS185-204VA) did not and cells transfected with this mutant receptor continued to proliferate in the presence of TGF- β (Fig. 5e). Thus, phosphorylation of receptor I by receptor II is an essential step in the TGF- β signalling pathway.

Discussion

Receptor-mediated signal transduction often involves ligand-dependent activation of multisubunit receptor complexes^{30,31,37}. The intracellular domains of these receptors, activated by the binding of ligand, then control the recruitment and activation of mediators of the intracellular signalling pathways. These pathways often lead to cytosolic kinase cascades. Here we describe the activation mechanism of the TGF- β receptor, a representative of the transmembrane Ser/Thr kinase class of receptors, and provide evidence for a novel mode of signal transduction involving two transmembrane kinases that act in sequence (Fig. 6).

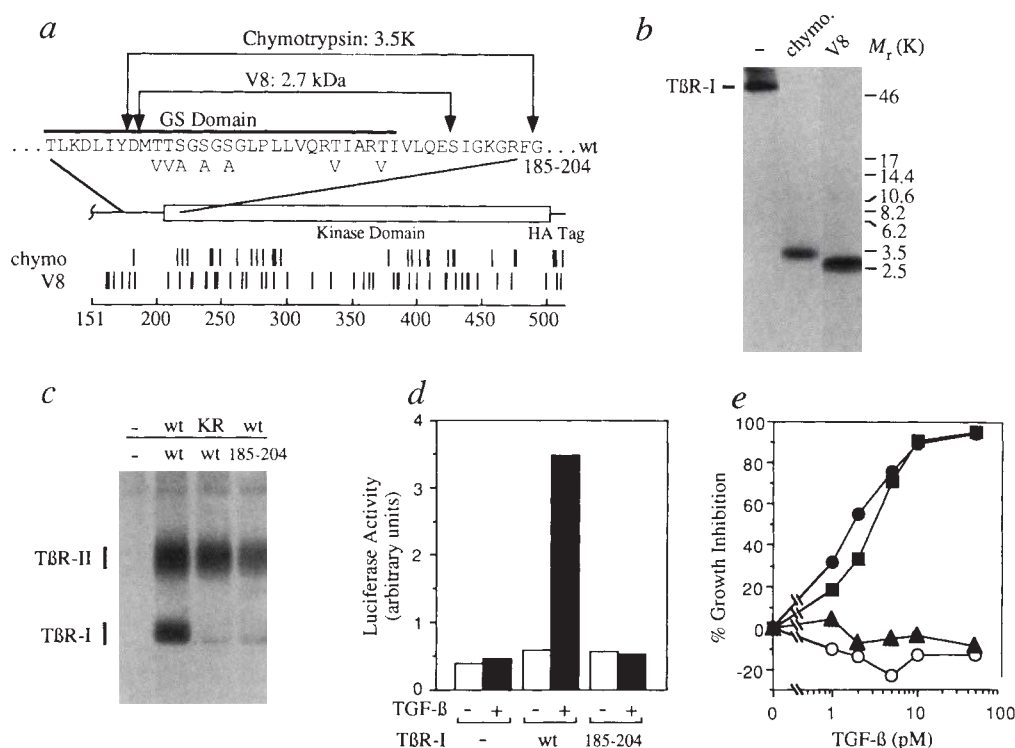
The TGF- β type II receptor binds ligand free in the medium which, by analogy with the tyrosine kinase receptors, raises the possibility that ligand binding activates its kinase domain. However, we show that T β R-II is constitutively autophosphorylated and ligand occupancy causes no appreciable change in this activity. Activin type II receptors also display ligand-independent autophosphorylation activity³⁸, suggesting that this is a general feature of type II Ser/Thr kinase receptors.

Unlike TGF- β type II receptors, receptor I at the cell surface is unable to bind ligand from the medium. However, receptor I can recognize ligand bound to receptor II and form a very stable ternary complex. The formation of this complex is tightly correlated with signalling²³. Cells defective in one or the other receptor are refractory to any TGF- β effects^{18,20,23,24,26,34,36}. The ability to label receptor I by crosslinking to 125 I-TGF- β using an 11-Å bifunctional reagent³⁹ indicates that receptor I contacts the ligand. Whether receptor I recognizes receptor II-induced conformational alterations in TGF- β or a TGF- β /receptor II protein interface is not yet known. Nevertheless, these data suggest that in the TGF- β receptor and presumably other Ser/Thr kinase receptors, the ligand does not function to activate the kinase domain of the primary receptor (receptor II) but rather functions to recruit type I receptors into a complex with type II receptors.

Recruitment of receptor I into the complex results in receptor I phosphorylation. This phosphorylation occurs on Ser and Thr residues and is not an autophosphorylation but rather a phosphorylation by receptor II. Proteolytic mapping of receptor I suggests that the GS domain is the site of phosphorylation. Receptor complexes in which receptor I is unphosphorylated either because of a mutation in the receptor II kinase domain or mutations in the GS domain, are unable to transmit signals. Thus, phosphorylation of receptor I by receptor II is essential for the propagation of TGF- β signals.

Functional analysis of T β R-I and T β R-II has shown that both kinase domains are essential for signalling all TGF- β responses^{20,26}. Because receptor I neither autophosphorylates nor phosphorylates receptor II, but is required for signalling, we conclude that receptor II functions to phosphorylate receptor I which propagates the signal to substrates downstream of the TGF- β receptor complex. Consistent with this model, the nature of the biological response to ligand in both the TGF- β and activin systems is specified primarily by the type I receptor isoform that is engaged in the complex^{17,26}.

FIG. 5 a, The primary sequence of the wild-type (wt) GS domain (over-line) and the changes introduced into the mutant T β R-I(TS185-204VA) (185–204) is indicated. The chymotryptic and V8 protease fragments generated from the GS domain (arrow brackets) and all the predicted chymotrypsin and V8 protease sites within the intracellular domain (vertical lines) are shown. The approximate amino-acid residue numbers of T β R-I are indicated on the scale at bottom. b, [32 P]phosphate-labelled T β R-I was incubated with chymotrypsin (chymo), V8 protease (V8) or no additions (–) and the resultant phosphopeptides analysed on Tris-Tricine gels⁴³. c, Receptor complexes were purified from [32 P]phosphate-labelled R-1B cells transiently transfected with the indicated T β R-II receptor (top row) and either wild-type (wt) or the GS domain mutant, T β R-I/HA(TS185-204VA) (185–204) (bottom row). d, e, T β R-I(TS185-204VA) does not restore TGF- β responses in receptor I defective cells. R-1B cells transiently transfected with the indicated T β R-I receptor and a TGF- β inducible luciferase reporter construct (3TP-Lux^{23,26}) were incubated with (+) or without (–) 250 pM TGF- β and cell lysates assayed in d as previously described^{23,26}. e, The TGF- β antiproliferative responses of Mv1Lu cells (filled circle), R-1B cells (empty circle) and R-1B cells stably expressing either T β R-I (filled square) or T β R-I(TS185-204VA) (filled triangle) were measured as previously described²⁶ and are plotted in e as the percentage decrease in [125 I]deoxyuridine



The phosphorylation of the GS domain in type I receptors could function in a number of ways to activate signalling by the kinase domain of type I receptors. Motifs in the intracellular domain of many different classes of receptors mediate signalling by regulating the association of signalling molecules with the activated receptor. By analogy, phosphorylation of the GS domain of receptor I could fulfil a similar function, acting as a docking site to bring substrates to the type I receptor. However, the GS domain of type I receptors that signal different biological responses are highly conserved^{1,21}. Thus, an alternative function

incorporation relative to untreated cultures²³. METHODS. Purified [32 P]phosphate-labelled T β R-I/HA was isolated from receptor complexes collected on Ni-NTA-agarose as described in Fig. 4. Phosphorylated T β R-I/HA was digested for 1 h at 37 °C in 50 μ l 50 mM ammonium bicarbonate containing 0.1 μ g of chymotrypsin 1 μ g V8 protease and the products were resolved on 17.5% Tris-Tricine gels⁴³. M_r standards are peptide fragments derived from myoglobin (Sigma).

for this region may be to act as a kinase activation domain analogous to the phosphorylated 'lip' identified in a number of cytosolic kinases⁴⁰. Suitable receptor substrates that could be used to distinguish between these possibilities have not yet been found. Thus, resolution of this issue awaits the identification of downstream targets of the type I receptors.

We have described the molecular events involved in activation of the TGF- β receptor. Whether every aspect of this mechanism will apply to all the Ser/Thr kinase receptors for the TGF- β superfamily awaits further study. However, the results suggest a general model for signal transduction by this class of receptors (Fig. 6), in which the ligand is directly involved in mediating the association of two distinct transmembrane kinases, which, with one phosphorylating the other, generates the first step in a signalling pathway.

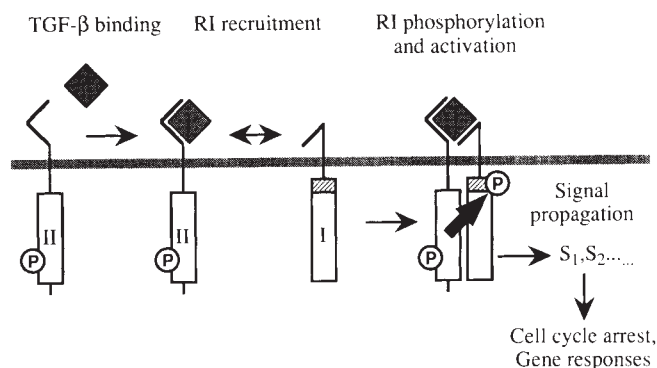


FIG. 6 A general model for the initiation of signalling by the TGF- β receptor. Receptor II is the primary TGF- β receptor and is a constitutively active serine/threonine kinase that recruits receptor I by means of bound TGF- β (diamond). Subsequent phosphorylation of the GS domain (striped box) by receptor II allows the receptor I kinase to propagate the signal to downstream substrates that mediate antiproliferative as well as gene responses.

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LETTERS TO NATURE

Detection of a large mass of dust in a radio galaxy at redshift $z = 3.8$

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ELLIPTICAL galaxies are thought to have formed most of their stars in a rapid burst in the early Universe¹, but an unambiguous example of a 'primaeval' elliptical galaxy (one undergoing its first major burst of star formation) has yet to be discovered. High-redshift radio galaxies are among the most promising candidates^{2,3}, because their low-redshift counterparts are identified exclusively with ellipticals, but the presence of an active nucleus complicates the analysis of their evolutionary state from optical-infrared observations^{3–5}. The failure of optical searches to detect primaeval ellipticals^{6–9} suggests that they may be very dusty, prompting us to search for thermal emission from the dust, which will be redshifted to submillimetre wavelengths in our reference frame. Our detection of submillimetre emission from the radio galaxy 4C41.17, reported here, suggests that it contains a large mass of dust, probably located in a dust lane obscuring the centre of the galaxy^{10–14}. The observations are consistent with the recent occurrence of a massive burst of star formation, but probably not the first such episode. We conclude that this galaxy was already in the final stages of its formation at a look-back time of 12–15 billion years.

Building on the discovery¹⁵ of far-infrared luminous starburst galaxies by the Infrared Astronomy Satellite (IRAS), we have embarked on a programme (D.H.H., J.S.D. and S.R., manuscript in preparation) to search for rest-frame far-infrared emission from high-redshift ($z > 2$) radio galaxies by observing that emission in the submillimetre range.

We observed 4C41.17, the most distant known galaxy¹⁰ (that is, the most distant known object with a spatially resolved optical continuum potentially dominated by starlight), as part of this programme on 30 September 1993. During exceptionally dry and stable conditions we were able to integrate continuously for three hours with the ³He bolometer UKT14 (ref. 16) on the

James Clerk Maxwell Telescope (JCMT) through the 800- μ m continuum filter. The observation was made using a beam size of 16.5 arcsec (equivalent to a linear size of 110–230 kpc, for cosmological density parameter $\Omega_0 = 1$ –0, and Hubble constant $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$), chopping at 7.81 Hz with an east–west throw of 60 arcsec. The result was a flux-density measurement of $S_{800 \mu\text{m}} = 17.4 \pm 3.1 \text{ mJy}$, the only unambiguous detection in a pilot study of six $z > 2$ radio galaxies. Further observations were made at 450 μ m in December 1993 (again in excellent conditions), resulting in a sensitive 3σ limit, $S_{450 \mu\text{m}} < 56 \text{ mJy}$, after a further three hours of integration.

On the basis of its rather blue ultraviolet–optical spectral energy distribution (SED) and complex multi-component morphology, it has been claimed that 4C41.17 may be a genuine example of a primaeval elliptical galaxy¹⁰. The importance of our 800- μ m detection is that it allows us to make a completely independent assessment of the 'evolutionary state' of 4C41.17.

Figure 1 shows the radio–ultraviolet SED of 4C41.17. Neither an extrapolation of the steep radio-lobe spectrum nor the much flatter spectrum of the recently discovered radio core ($\alpha = -0.23$, where α is the spectral index for the flux density, $f_\nu \propto \nu^\alpha$) can account for the observed 800- μ m flux density. Our detection can thus only be explained by thermal emission from dust or by non-thermal emission from an additional synchrotron component. Although our data do not allow us to exclude the second of these possibilities on the basis of the submillimetre spectral index ($\alpha > 2.5$) it is highly implausible that the far-infrared emission of 4C41.17 is due to a synchrotron component. To avoid exceeding the observed flux-density of the radio-core at the observing wavelength $\lambda_{\text{obs}} = 2 \text{ cm}$, this component would need to become self-absorbed at the rest-frame wavelength $\lambda_{\text{rest}} < 510 \mu\text{m}$; such compact ($< 0.015 \text{ pc}$) and energetic synchrotron components have only ever been observed in the flaring 'blazar' class of active galactic nuclei, where they are always accompanied by a series of components with progressively lower turnover frequencies, producing the characteristic flat radio spectrum. It is therefore hard to sustain a synchrotron interpretation of our 800- μ m data, as 4C41.17 would then be unique in containing, in isolation, such an energetic and compact emission region.

It is therefore reasonable to assume that the rest-frame far-infrared emission in 4C41.17 is dominated by optically thin thermal emission from dust, particularly as this assumption has been validated for IRAS-detected galaxies (such as F10214 + 4724; refs 17, 18) and radio-quiet quasars^{19,20}, which, like 4C41.17, have far-infrared peaks in their rest-frame SED. The addition of a 450- μ m limit to the 800- μ m detection restricts the rest-frame dust temperature to $\leq 43 \text{ K}$ (for $\beta = 2$) or $\leq 67 \text{ K}$ (for $\beta = 1$), where β is the grain emissivity index. Adopting a dust temperature $T_{\text{dust}} = 50 \text{ K}$, $\Omega_0 = 1$, $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and an average dust opacity (at a rest-frame wavelength of $167 \mu\text{m}$) of $\kappa_d = 3.5 \text{ m}^2 \text{ kg}^{-1}$ (refs 21, 22), yields a dust mass $M_{\text{dust}} \approx 3 \times 10^8 M_\odot$.

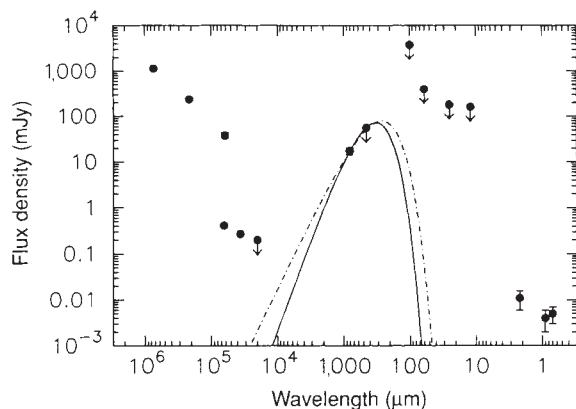


FIG. 1 The observed spectral energy distribution of 4C41.17 from radio to optical (rest-frame ultraviolet) wavelengths. The figure shows the radio flux densities of both the steep-spectrum lobes^{10,12} (at $\lambda = 7.35 \times 10^5$, 2×10^5 and $6.38 \times 10^4 \mu\text{m}$), and also the flat-spectrum core¹² (at $\lambda = 6.38 \times 10^4$, 3.6×10^4 and $2 \times 10^4 \mu\text{m}$). Extrapolation of either of these radio components to a wavelength of $800 \mu\text{m}$ produces a non-thermal contribution which is ~ 100 times smaller than the observed $800\text{-}\mu\text{m}$ flux density. It is therefore almost certain that the observed submillimetre flux density is produced by thermal emission from dust. The solid curve shows the emission expected from dust of emissivity index $\beta = 2$ at a temperature of $T_{\text{dust}} = 43 \text{ K}$ normalized to our $800 \mu\text{m}$ detection. The dashed-dotted curve represents the thermal emission where $\beta = 1$ and $T_{\text{dust}} = 67 \text{ K}$. Also shown are 3σ IRAS limits at $100 \mu\text{m}$ and $60 \mu\text{m}$ (derived from direct determination of the noise level in IRAS maps of the source region), the continuum flux density observed at $2.2 \mu\text{m}$ (from the line-free K_s image¹⁴) and the (uncorrected) I -band and R -band flux densities¹⁰.

The dust mass in 4C41.17 is ≥ 10 times greater than is typically found in low-redshift radio galaxies²³; adopting the same model parameters as used by Knapp and Patten²³ ($T_{\text{dust}} = 18 \text{ K}$ (not excluded by our data), $\kappa_d = 1.5 \text{ m}^2 \text{ kg}^{-1}$ at $\lambda_{\text{rest}} = 167 \mu\text{m}$, and $H_0 = 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$) yields a dust mass in 4C41.17 of $4.0 \times 10^9 M_\odot$. The dust mass is also several times greater than that inferred for the most extreme local ($z = 0.44$) cD galaxy 09104+4109 (ref. 24), or radio-loud quasars and galaxies at $z \approx 1$ (ref. 25). Compared to elliptical galaxies which have been studied in the rest-frame, far-infrared 4C41.17 is clearly exceptionally luminous.

Because of the suppression of the resonant Lyman- α line expected in dusty environments, the huge Ly- α luminosities of radio galaxies like 4C41.17 appear at first sight to be inconsistent with such large dust masses. In the light of our JCMT detection we have therefore re-examined the Fabry-Pérot Ly- α image of 4C41.17 (ref. 13) to look for the most likely location of the dust. In fact, there is a clear dip in the surface brightness of the $\sim 15 \times 10 \text{ arcsec}$ Ly- α halo centred 2.2 arcsec west of the Ly- α peak—a dip which the observers chose to associate with a foreground Ly- α absorber, unrelated to the radio galaxy. Given that its position is now known to coincide with that of the recently discovered radio core of the galaxy¹², we prefer to interpret this dip as resulting from absorption of the back half of the Ly- α halo by a dusty torus of approximate dimensions $10 \times 30 \text{ kpc}$ (for $\Omega_0 = 1$, $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$) oriented perpendicular to the radio jet axis, and centred on the radio core (see Fig. 2). This agrees quantitatively with the observed line profiles if the Ly- α halo is expanding, as would be expected if it is driven by the radio-source bowshock¹⁰: the blue wing of the line is similar at the spatial peak of the Ly- α and at the site of the absorber, whereas at the redder line centre, the surface brightness drops by a factor ~ 3 . Moreover, as we show in Fig. 2, this putative dust lane corresponds exactly with pronounced gaps in the rest-frame ultraviolet¹¹ and optical¹⁴ images. Regardless of the detailed geometry, the lack of other holes in the Ly- α image

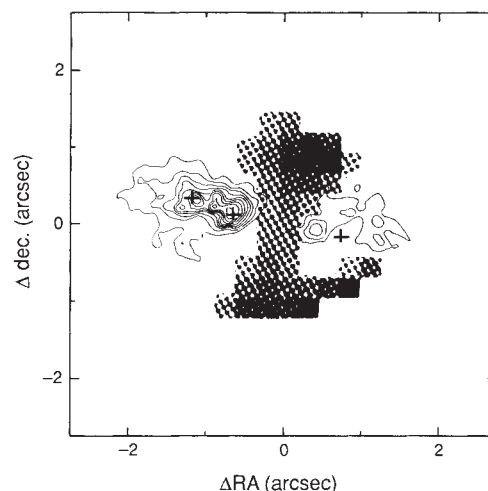


FIG. 2 The probable location of dust in 4C41.17. A grey-scale image of the region of Ly- α obscuration is shown (reproduced from Hippelein and Meisenheimer¹³), superimposed on a suitably scaled and registered (accurate to $\sim 0.25 \text{ arcsec}$) contour plot of the Hubble Space Telescope image obtained by Miley et al.¹¹ ($\lambda_{\text{rest}} \approx 1,400 \text{ Å}$). The image is centred on the coordinates of the recently discovered¹² flat-spectrum core of the radio galaxy—right ascension (RA) 06 h 47 min 20.574 s, declination (dec.) $41^\circ 34' 3.85''$ (B1950); for our $800\text{-}\mu\text{m}$ observation the 16.5-arcsec aperture of the JCMT was centred at RA 06 h 47 min 20.74 s, dec. $41^\circ 34' 4.5''$. Our interpretation of this region of Ly- α obscuration as a dust lane in the radio galaxy is supported by the obscuration overlying the position of the radio core, and by its location coinciding almost exactly with a distinct gap in the ultraviolet continuum emission. In addition, it appears to be orientated perpendicular to the radio axis. The three crosses indicate the positions of the radio components B1, B2 and B3¹⁰⁻¹².

gives us confidence that the huge dust mass detected within the 16.5-arcsec JCMT aperture is associated with the inner regions of the radio galaxy itself, and not with a companion or foreground object. Our preferred geometry for the dust in 4C41.17 has potentially far-reaching implications: if all young galaxies have a high dust content, then detections of a Ly- α line may be feasible only when it is produced away from the dusty starburst region by processes associated with the active nucleus.

Is 4C41.17 a primeval elliptical galaxy? By analogy with IRAS-detected galaxies, and assuming that the dust is heated primarily by young stars, we can use the far-infrared luminosity of 4C41.17 ($L_{\text{FIR}} \geq 5 \times 10^{13} L_\odot$; $\Omega_0 = 1$, $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$), to estimate the 'current' star formation rate (SFR). A simple scaling between SFR and far-infrared luminosity, calibrated using nearby starbursts such as M82 (ref. 26), yields an estimated SFR of $(2\text{--}10) \times 10^3 M_\odot \text{ yr}^{-1}$ ($\Omega_0 = 1\text{--}0$). These values agree rather well with the SFR ($\sim 4,300 M_\odot \text{ yr}^{-1}$) inferred from the very young (10^8 yr), dusty model (model D) fit to the ultraviolet-optical SED of 4C41.17 (ref. 10). More generally, star formation rates of $\sim 1,000 M_\odot \text{ yr}^{-1}$ are obviously consistent with an extremely luminous giant elliptical galaxy (with a stellar mass of $\sim 10^{12} M_\odot$; ref. 27) observed during a canonical 1-Gyr formation starburst.

However, any attempt to determine whether a galaxy is primeval on the basis of its far-infrared luminosity is vulnerable to the same criticisms as arguments based on the shape of the ultraviolet-optical SED. First, although there is no obvious quasar nucleus in 4C41.17, it may well be hidden from view by the very dust detected by our JCMT observation. (In fact, this seems highly likely, given that the dust appears to cover the nucleus as shown in Fig. 2). If this is true, then the dust may be heated by this buried quasar and not by a starburst, in which case the SFR could be very much lower than is deduced by comparison with starburst galaxies. Second, even if correct, both the high

SFR inferred from the far-infrared luminosity of 4C41.17 and the shape of the ultraviolet-optical SED are still consistent with an old galaxy (with formation redshift $z_f > 10$) undergoing a 10^6 -yr starburst involving as little as 1% of the mass of the underlying galaxy.

Similar problems afflict attempts to determine the evolutionary state of 4C41.17 from its peculiar optical morphology. The rest-frame wavelength of the Hubble Space Telescope image reproduced in Fig. 2 is 1,400 Å, and even at $z < 1$ it is now well established that powerful radio galaxies frequently possess a component of ultraviolet light aligned with the radio structure⁴. Our conclusion that the centre of the galaxy is concealed by a dust lane further weakens claims that its multi-modal optical structure is indicative of an elliptical galaxy in the process of formation. It also remains difficult to rule out the presence of a significant population of evolved stars, as even near-infrared observations of 4C41.17 (ref. 14) sample continuum light at a rest-frame wavelength of only 4,600 Å.

In principle, therefore, the most straightforward way to identify whether a galaxy at $z > 3$ is genuinely young is to determine what fraction of its final stellar mass has yet to be converted into stars at the epoch of observation. 4C41.17 certainly contains much more dust than has been found in low-redshift radio galaxies. Although the uncertainties involved in converting this dust mass into a gas mass are large (D.H.H., J.S.D. and S.R., manuscript in preparation), assuming a canonical gas to dust ratio of 500, which is supported by recent CO observations of 10214+4724 (ref. 28), leads to an estimate of the gas mass in 4C41.17 of $M_{\text{gas}} \approx 10^{11} M_{\odot}$. This value is at least consistent with the idea that a significant fraction ($\sim 10\%$) of the galaxy's eventual stellar mass has yet to be converted into stars at $z = 3.8$.

Thus, although our detection of dust in 4C41.17 indicates the occurrence of recent large-scale star-formation activity, it does not prove that 4C41.17 is 'primordial'. Indeed, taken at face value, our results indicate that we are observing a massive starburst in 4C41.17 involving ~ 5 – 10% of its eventual stellar mass, as would be expected for a giant elliptical galaxy in the final stages of formation. None of the available data on 4C41.17 can yet distinguish whether star formation in 4C41.17 began at $z \approx 4$, or whether the bulk of its stellar content was formed at much higher redshifts. It remains entirely possible that the general population of elliptical galaxies was formed at $z > 10$, and that in high-redshift radio galaxies at $z \approx 3$ – 4 we are observing the final spectacular stages in the construction of luminous cD galaxies by mergers. □

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Density and size of comet Shoemaker–Levy 9 deduced from a tidal breakup model

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ALTHOUGH comets have been studied throughout most of recorded history, a detailed understanding of their internal properties is still lacking. Recent observations¹ of the split comet Shoemaker–Levy 9—actually a spectacular string of cometary fragments that resulted from the tidal disruption of a single parent body as it passed close to Jupiter^{2–5}—have therefore stimulated much interest, as they provide an unprecedented opportunity to investigate the physical properties of comets more generally^{6–8}. I report here simulations of the tidal breakup of the parent comet, which I assume to have been an assemblage of a large number of spherical components bound together only by gravity. Following the initial tidal disruption of the assemblage, the particles coalesce rapidly by mutual gravitation into a chain of larger fragments, the morphology of which depends critically on the density of the components. By comparing the size, number and distribution of the simulated fragments with observations of Shoemaker–Levy 9, I determine an average comet density of about 0.5 g cm^{-3} and a parent comet diameter of about 1.8 km.

In contrast to the common view of a solid body⁹, I model the comet as an agglomeration of individually competent components—'snowballs'—bound together only by mutual gravitational attraction. The depiction of comets as 'flying rubble piles' has enjoyed increasing support^{6,10,11} and comets with multiple nuclei are not exceptional^{12–14}. There are probably other cohesive forces between components, but I assume that these are small compared to gravitational binding.

In the simulations, the spherical components interact gravitationally except when they touch. The touching, or collision, of two components is handled as a non-adhesive dissipative scattering, that is, the velocities are suddenly changed in such a way that momentum is conserved, but some of the kinetic energy is converted to heat. The simulation is a detailed calculation of the gravitational interaction and collisions of the components—it is not a hydrodynamic calculation.

A further simplification, which greatly accelerates computation, is to assume that the radius r_0 and density ρ of each component is the same. Under this assumption, the equation of motion in the vicinity of the comet's centre of mass is well approximated by

$$\ddot{\mathbf{r}}_j = G \left[m_0 \sum_{i \neq j} \frac{\mathbf{r}_i - \mathbf{r}_j}{|\mathbf{r}_i - \mathbf{r}_j|^3} + \frac{2M(\mathbf{R} \cdot \mathbf{r}_j)\mathbf{R}}{R^5} \right] \quad (1)$$

where G is the universal gravitation constant, M is the jovian mass, m_0 is the component mass, $\mathbf{r}_i$ is the radius vector of the i th component from the comet's centre of mass, and $\mathbf{R}$ is the radius vector of the comet's centre of mass from the jovian



FIG. 1 *a-f*, Sequence of calculated configurations for the comet started at 2×10^4 s before perijove. *a*, Perijove; *b*, initial breakup of the comet 10^4 s after perijove; *c*, 2×10^4 s, coalescence is starting; *d*, 3×10^4 s, major fragments are forming; *e*, 4×10^4 s; *f*, 5×10^4 s, most of the morphology is established.

centre. The first term of equation (1) is the net gravitational acceleration due to all the other components and binds the comet together. The second term is the acceleration due to the gradient in Jupiter's gravitational field and represents the tidal forces that pull the comet apart: components on the Jupiter side of the comet's centre of mass move toward the planet, whereas those on the opposite side move away.

As long as all the components remain separated by at least two radii, the motion is found by straightforward integration of equation (1). A 'collision' occurs whenever $|\mathbf{r}_i - \mathbf{r}_j| < 2r_0$ and the emergent velocities are given by

$$\dot{\mathbf{r}}'_i = \dot{\mathbf{r}}_i - \frac{\delta(\dot{\mathbf{r}}_i - \dot{\mathbf{r}}_j) \cdot (\mathbf{r}_i - \mathbf{r}_j)}{8r_0^2} (\mathbf{r}_i - \mathbf{r}_j) \quad (2)$$

A frictionless collision can only alter the normal component of

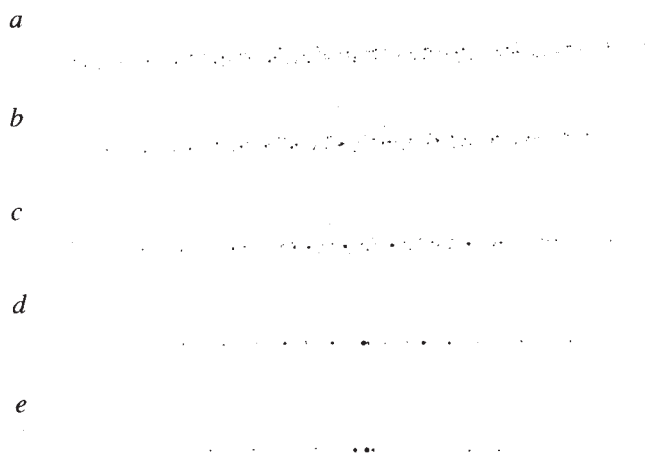


FIG. 2 *a-e*, Dependence of breakup on density of components. Configurations are shown at 10^5 s after perijove when size and relative position of the fragments are well established. Values of density (ρ) as follows (all g cm^{-3}): *a*, 0.45; *b*, 0.50; *c*, 0.55; *d*, 0.60; *e*, 0.65.

the relative velocity. If $\delta = 2$, the normal component of the relative velocity simply reverses direction and the collision is perfectly elastic. If $\delta = 1$, the normal component is reduced to zero in the collision. It is easy to see that the only allowed values are $1 \leq \delta \leq 2$.

We have little knowledge of how components of this sort might lose kinetic energy in collisions. For this calculation, the details are not very important. It can be shown that for completely random impact parameters, the selection of $\delta = 1$ causes the average collision between components to lose half its relative kinetic energy to heat. This seems realistic. Because the gravitational orbital dynamics favour grazing collisions over random impact parameters, $\delta = 1$ will result in slightly less than half energy loss on average.

The model embodied in equations (1) and (2) enjoys a remarkable scaling relationship: all distances scale with simple similarity. Locations are described by the dimensionless vector $\mathbf{r}_i/r_0$. If we increase the diameter of the comet by a factor of 2, the geometrical arrangement of all components at any time after disruption will be exactly the same, with the distance scale increased by a factor of 2. The energetics enjoy a similarly simple scaling relation. A factor of 2 increase in component radius increases all energies (kinetic energy, gravitational potential energy and heat generated in component collisions) by a factor of $2^5 = 32$. As a result of these scaling properties, we can cover comets of all sizes with a single calculation.

For the initial geometrical arrangement, I place one component at the centre of mass with 320 components packed around it in a face-centred cubic (f.c.c.) array, which results in a comet model that is close to a gravitational potential minimum. The time step for the dynamical calculation is adjusted so only binary collisions occur, although there may be many binary collisions among separate pairs within that time step. The lattice spacing for the spheres to just touch is $r_0/\sqrt{2}$, but this contact packing would cause the binary-collision condition to be violated on the first time step. So I use an initial lattice spacing of $r_0(\sqrt{2} + 0.0001)$ —spheres are very close together, but not actually touching.

I approximate the encounter of Shoemaker-Levy 9 with Jupiter as a parabolic orbit with a closest approach (perijove) distance of 9.46×10^4 km (1.36 jovian radii) from the centre of the planet¹⁵. The orbit is obtained by integrating the newtonian equations of motion in the usual manner and describes the time-dependence of the location of the comet's centre of mass. Figure 1 shows the early stages of breakup as the comet passes Jupiter. Figure 1*a* depicts the comet at perijove and the directions of the cartesian coordinates. The orbital plane is parallel to the x - y plane and the x axis is parallel to the parabolic line of symmetry. The configurations are 10^4 s apart. The components have a density $\rho = 0.55 \text{ g cm}^{-3}$ and are shown to scale. The sequence shows the initial pulverization of the comet by the tidal forces and subsequent coalescence into larger fragments by mutual gravitation.

Keeping the initial geometry the same, the fate of the comet is crucially dependent on its density. This sensitivity allows us to estimate the density from the comet's qualitative behaviour. For high densities, the binding term in equation (1) is always large compared to the tidal term, and the comet will not break up. For low densities, the comet will break into individual components, which simply disperse and never coalesce into larger objects. Figure 2 shows different breakup configurations resulting from identical initial geometry but different density. Each case is shown at 10^5 s after perijove, and with perspective approximately as it would be viewed from Earth. (Further coalescence and motion among the fragments result in only small morphological changes after 10^5 s.) At later times, the chain becomes long compared to the fragment size, so that it cannot be shown to scale. Because observational brightness is roughly proportional to cross-sectional area, I have chosen to make the comparisons at this relatively early time when the cross-sections

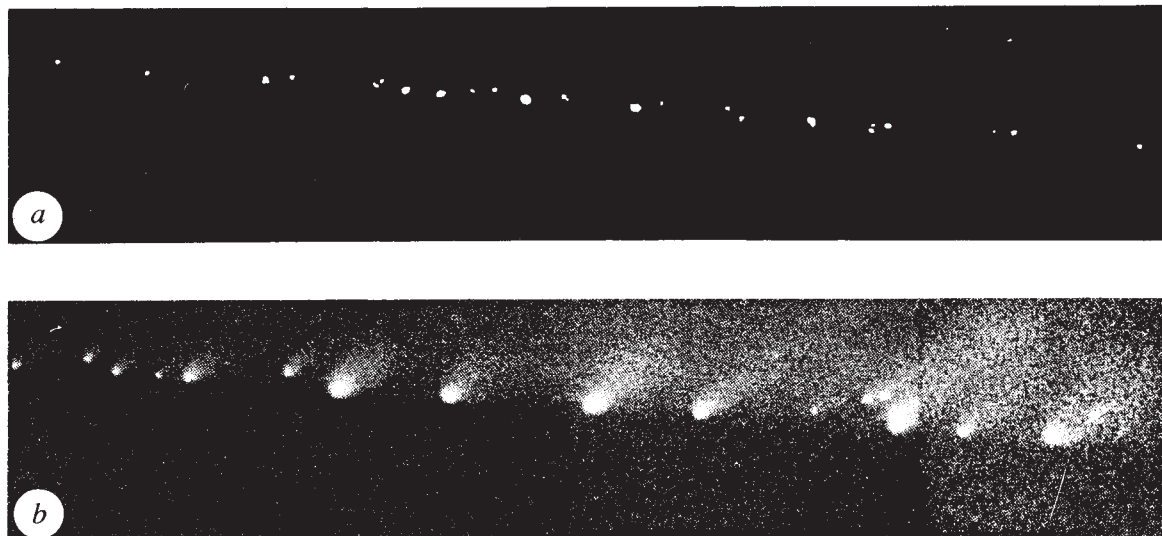


FIG. 3 *a, b*, Comparison between the brightness simulation from calculated breakup configuration shown as viewed from Earth at

10^6 s (*a*) and the observation of Shoemaker–Levy 9 by the Hubble telescope (*b*).

are still clearly visible. Ultimately, the length of the chain will increase with time t as $\sim t^{4/3}$ while its width will increase as $\sim t$. The chain will become thinner, but the size of the fragments and their relative spacing along the chain will remain the same.

Figure 2c is for $\rho = 0.55 \text{ g cm}^{-3}$ and corresponds most closely to the observed morphology of the comet chain. Figure 3a is a simulation of the relative brightness that would be observed from Earth for the $\rho = 0.55 \text{ g cm}^{-3}$ case at 10^6 s after perijove. The morphology is very similar to Fig. 2c, but some further coalescence has taken place. There is no coalescence between 5×10^5 s and 10^6 sec. Figure 3a shows a total of 23 fragments with 7 or 8 major fragments, in good agreement with the general description given of Shoemaker–Levy 9. Figure 3b is a reproduction of a recent Hubble observation¹⁶. The comparison is necessarily somewhat subjective.

Figure 2 suggests that the components have a density between 0.5 and 0.6 g cm^{-3} . The initial packing on an f.c.c. lattice makes the starting object a bumpy sphere. About 11% of the interior of the bumpy sphere is void, so the initial density of the comet is $\sim 0.5 \text{ g cm}^{-3}$.

For the calculations thus far presented, I have chosen $\delta = 1$. For $\delta = 2$, collisions between components are perfectly elastic, and once the components are dispersed by the tidal forces, they will never coalesce into larger fragments. A numerical simulation midway between the extremes, $\delta = 3/2$, gives a final configuration similar to the $\delta = 1$ case. The configuration can be brought into closer correspondence by a slight increase in density. The reason for the relative insensitivity is in part connected with the small fraction of energy that goes to heat.

The final configuration is also not very sensitive to the number of components in the initial configuration. Comets with larger numbers of components convert kinetic energy to heat more quickly, the rate going approximately as the one-third power of the number. A numerical simulation with 177 components produces a configuration qualitatively similar to the 321-component case, perhaps looking rather more like Fig. 2b. A broader study of the variation shows that a substantial increase in the number of components can be compensated by a slight decrease in density.

Initial rotation of the comet can have a more serious effect on the morphology. The angular velocity at which the gravitationally bound comet will be torn apart by centrifugal force is $\omega_c \approx \sqrt{Gm/r^3}$, where m is the comet's mass and r is its radius.

So ω_c sets an upper limit on the rotational speed. From numerical simulations, I find that an initial angular velocity of $\omega_c/3$ will introduce a density-estimate uncertainty of $\pm 0.05 \text{ g cm}^{-3}$, which is as much accuracy as I could claim from the qualitative comparison anyway. Thus if the initial angular velocity of Shoemaker–Levy 9 is $< \omega_c/3$, the quantitative results given in this Letter should stand. We have very little information on the rotation of comets, but sparse information on asteroids shows few rotating near their centrifugal limit.

Because of the scaling of equations (1) and (2), I can very roughly estimate the initial size of the comet from the length of the string of fragments. For a parabolic orbit at late times, it can be shown that $|\mathbf{r}_k - \mathbf{r}_l| \propto t^{4/3}$, where k and l are the furthest separated components. At 10^6 s after perijove, the numerical calculation gives $|\mathbf{r}_k - \mathbf{r}_l| = 2.4 \times 10^4 r_0$ and the 321-component f.c.c. bumpy sphere has a greatest diameter of $16.42 r_0$. On 1993 March 26.3 (2.26×10^7 s after perijove when the orbit was still reasonably parabolic) the string length was observed to be $1.64 \times 10^5 \text{ km}$, which implies $r_0 = 1.1 \times 10^{-1} \text{ km}$ and an initial diameter of 1.8 km, somewhat smaller than the diameter obtained by Scotti and Melosh⁶ using a perijove distance of 1.57 jovian radii.

Note added in proof: Using a related model that included gravity but no collisions, Asphaug and Benz (*Nature* **370**, 120–124, 1994) obtained similar density constraints for the parent comet. □

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Identity of planar defects in the 'infinite-layer' copper oxide superconductor

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The 'infinite-layer' compound¹⁻³ ACuO_2 (where A stands for cations such as strontium or calcium), has the simplest structure of all superconducting copper oxides, with only bare cations separating the CuO_2 planes. Accordingly, an understanding of the doping mechanism(s) that lead to superconductivity in this compound may facilitate the elucidation of the same phenomenon in the other copper oxide superconductors. Recently, Azuma and co-workers^{2,4} observed planar defects in an infinite-layer phase synthesized at high oxygen pressure, and proposed that the defects are A-cation deficient, and lead to superconductivity (with transition temperature $T_c \approx 100\text{--}110\text{ K}$) in this compound. Here, based on quantitative X-ray and high-resolution electron-microscopic analysis of the planar defects in $(\text{Sr}, \text{Ca})\text{CuO}_2$, we propose that the defects consist of a corrugated Sr-O layer substituted for a CuO_2 layer, with the incorporation of apical oxygen atoms (which are absent in the parent structure) at roughly half the available sites in the neighbouring Sr layers. This is equivalent to an insertion of a $\text{Sr}_3\text{O}_{2+x}$ block in an otherwise infinite-layer sequence. The variable oxygen stoichiometry of our defect model can account for the occurrence of p-type superconductivity (following high-pressure oxygenation), n-type superconductivity (high-pressure reduction) or lack of superconductivity (high-pressure neutral-atmosphere annealing) in this system, depending on the synthesis conditions⁴.

Infinite-layer samples with the nominal chemical formula $(\text{Sr}_{1-x}\text{Ca}_x)_y\text{CuO}_2$ ($y=0.9, 1.0$ and 1.1 ; $x=0.3$) were prepared using pre-synthesized precursors of low-pressure forms of $(\text{Sr}_{1-x}\text{Ca}_x)\text{CuO}_2$ with additions of CuO and $(\text{Sr}_{1-x}\text{Ca}_x)_2\text{CuO}_3$, followed by a high-pressure treatment. The synthesis conditions were 6 GPa oxidizing pressure at $1,050^\circ\text{C}$ for 1 h. The oxidizing pressure was created in a classical piston-cylinder press by decomposition of KClO_4 , which sandwiched the sample pellet; the 'sandwich' was contained in a gold capsule. All the compositions exhibited an onset of superconductivity above 100 K with varying Meissner fractions. (We have since synthesized a superconducting infinite-layer compound without calcium (that is, just SrCuO_2) but the synthesis conditions are not yet optimized for high Meissner fraction.)

Neutron and X-ray powder diffraction data showed that the dominant phase was the infinite-layer structure, with peak broadening characteristic of planar defects. No other phases known to be superconducting were seen. In particular, there was no evidence of $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+x}$ -type phases, which are formed under similar high-pressure conditions and can have T_c s near 100 K (refs 5, 6).

High-spatial-resolution X-ray microanalysis was performed using a cold field-emission gun transmission electron microscope (Hitachi HF-2000) equipped with an Oxford Pentafet thin-window X-ray detector⁷. A probe size of $\sim 1\text{ nm}$ full-width half-

maximum (FWHM) was positioned on the planar defects, and X-ray emission spectra from these regions were compared with those obtained from defect-free regions. In all cases, care was taken to account for spurious signals, counting statistics and appropriate Student's *t*-test error analysis⁷.

Dedicated imaging was performed using a Hitachi H-9000 high-resolution electron microscope (HREM). Initially, the high-resolution images were compared visually to simulated ones using two multislice routines (NUMIS (L. D. Marks, Northwestern University, USA) and the MacTempas suite of programs (Total Resolution, Berkeley, USA)) as a function of objective lens defocus and specimen thickness. A second more careful set of experimental images were then obtained, digitized and compared quantitatively with calculated images. Briefly, the standard deviations of the experimental data were obtained from the cell-to-cell variations, and these values used for conventional χ^2 fittings (where χ^2 is defined as the total sum of the square of the difference between the experimental and simulated value for each pixel; the reduced χ^2 is normalized by the number of the data points). The electron-optical conditions were determined by fitting the matrix, and these same conditions were used for the defect structure. The atomic positions of the defect structure were determined by minimizing the χ^2 error.

The planar defects that we observed in our sample are virtually identical in terms of appearance, contrast, form and distribution to those reported earlier^{2,4}. Some of the planar defects do not extend indefinitely in the x - y plane, but gradually disappear in the bulk crystal. The defect density varies from region to region. The X-ray microanalysis results (Fig. 1) with a 1-nm probe show an excess $(\text{Sr} + \text{Ca})/\text{Cu}$ X-ray signal from the planar-defect regions. A notable decrease in the Ca signal in the defect region was observed, but overall, there was an enrichment of the A-cation $(\text{Sr} + \text{Ca})$ relative to Cu in the defect region. The composition of the defect region (which includes some non-defective material because of beam broadening) was calculated using the non-defective region as an internal standard. Relative to the non-defective region, there was an excess of $\sim 32\%$ Sr/Cu atomic ratio, depletion of $\sim 27\%$ Ca/Cu atomic ratio, but excess of $\sim 16\%$ A-cation $(\text{Sr} + \text{Ca})$ over Cu on the planar defect. X-ray beam-broadening calculations and Monte Carlo simulations⁸ indicated that the excess of A-cation to Cu translates into substitution of a single layer of Sr (and some O) for a Cu layer, and considerable depletion of Ca in the defect region, such that the overall chemistry of the defect region is cation-rich. In addition to the nanoprobe analysis, a larger probe ($\sim 50\text{--}100\text{ nm}$ FWHM) was positioned in areas containing closely spaced ($\sim 2\text{--}5\text{ nm}$)

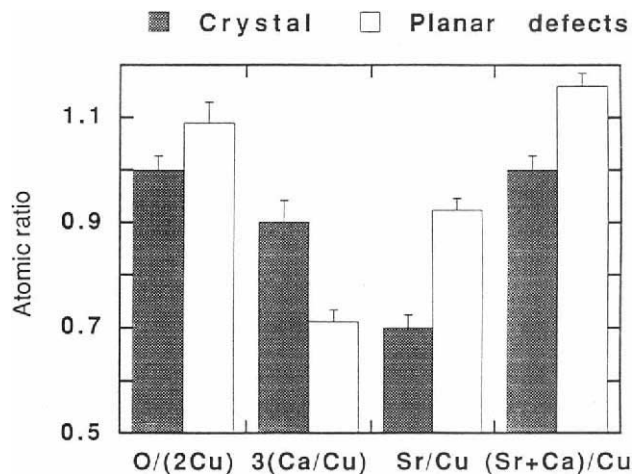


FIG. 1 Energy dispersive X-ray analysis of planar defects and perfect crystal with $\sim 1\text{-nm}$ electron probe. Error bars show the standard error of the mean, determined from 10 sets of measurements both on and off the planar defects.

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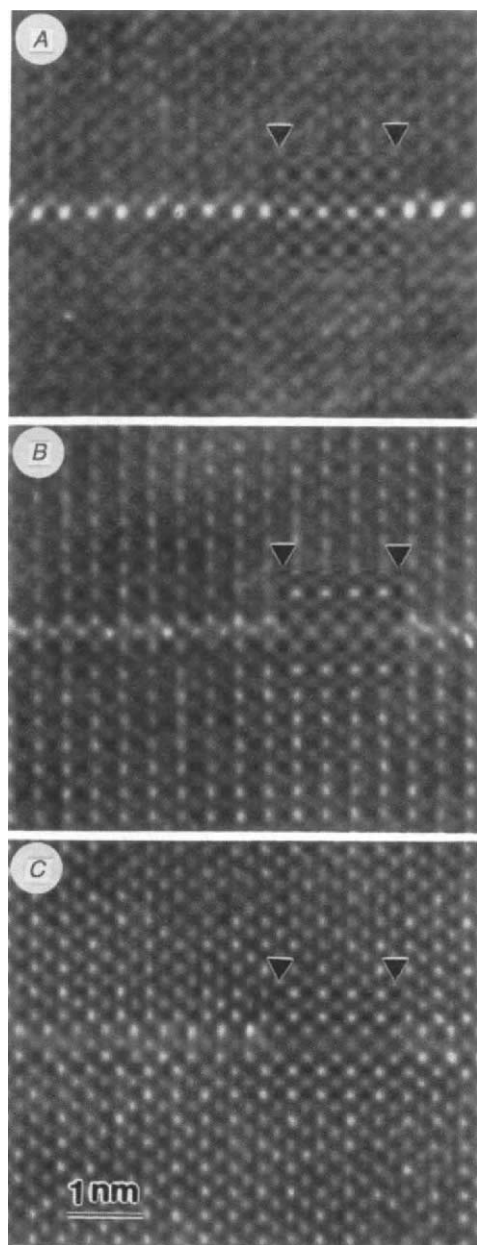


FIG. 2 Comparison of experimental and simulated HREM images of planar defects in the infinite-layer compound as a function of objective lens defocus. The defocus distances are 0, -440 and -740 Å for panels A, B and C, respectively, with a sample thickness of 20 Å. The reduced χ^2 values are 2.01 (A), 1.72 (B) and 1.66 (C).

planar defects, and the X-ray data compared with that obtained with the same probe size in defect-free regions. Consistent excess of (Sr + Ca) over Cu in the defect-rich region was also observed in these experiments. These results contradict the A-cation deficiency model proposed by Hiroi *et al.*^{2,4}

Exploiting the X-ray microanalysis results, a large number of different structural models were tested against experimental HREM images. Figure 2 shows three images. Analysis of the images indicates that changes need to be made both in the CuO₂ plane and in the Sr planes above and below the defect layer. Simply substituting Sr for Cu for this plane and/or introducing O vacancies did not reproduce the experimental images. We also tested the cation-deficiency model proposed by Hiroi *et al.*⁴ and reached the same conclusion: our simulated images of their model deviate strongly from our high-resolution images as well as theirs.

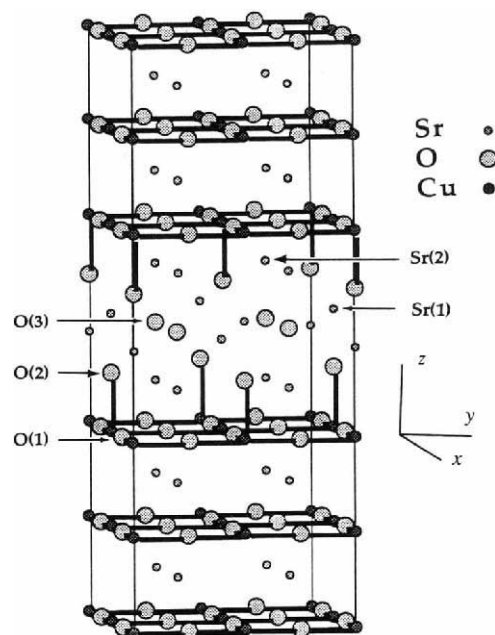


FIG. 3 The structural model for the planar defects, two unit cells wide along x and y directions. Three Sr-O layers replace a Sr-CuO₂-Sr block in an otherwise perfect infinite-layer structure. The ordering in the figure is shown only for convenience and to aid the eye.

The structural model that matches all the experimental HREM images is shown in Fig. 3. The reduced χ^2 values are 2.01, 1.72 and 1.66 for defoci of 0, -440 and -740 Å, respectively, as shown in Fig. 2A, B and C. This model is A-cation (Sr) rich, with the substitution of a corrugated Sr-O layer for a CuO layer, and the incorporation of oxygen at roughly half the available sites in the neighbouring Sr layers (that is, substitution of an Sr₃O_{2±x} block for a Sr-CuO₂-Sr block in an otherwise infinite-layer structure sequence). The middle layer consists of a stoichiometric Sr(1)-O(3) sheet, whereas the two outer Sr-O layers, which contain Sr(2) and O(2), have ~50% occupancy of O(2). The Sr(1) atoms are not coplanar with the O(3) atoms, but are staggered up or down from the centre of the unit cell by $0.58 \pm 0.07(\sigma)$ Å along the c-axis (the mean and error for vertical staggering are calculated based on fitting data from three defocus measurements). Because HREM images or diffraction patterns taken along other zone axes (for example, [110]) did not show any higher-order periodicity for the defects, we assumed random occupancy for Sr(1): in other words, the sign of the staggering (above or below the centre of the unit cell) of Sr(1), and thus that of O(2), may vary. The distance from the centre of the planar defect to the neighbouring CuO₂ planes (through a Sr(2)-O(2) layer) is increased from 3.4 Å to ~3.7 Å, in agreement with the previous observations⁴.

Based on charge-neutrality arguments, about half of the possible sites for O(2) contain oxygen, leading to apical oxygen atoms for about half of the Cu atoms in the adjacent CuO₂ planes, located along the longer Sr(1)-Cu bond. For the Sr(2) position, the simulation indicates that the Sr(2) atom is $1.5 \pm 0.1(\sigma)$ Å away from the nearest CuO₂ plane. The Sr-O distances are; Sr(1)-O(2) = 2.13 Å, Sr(1)-O(3) = 2.82 Å, Sr(2)-O(2) = 2.84 Å and Sr(2)-O(3) = 2.22 Å. The atomic positions for the defects are listed in Table 1.

Our model for the planar defects is a configuration not yet seen in any layered copper oxide compounds made under ambient conditions. The similarity to the Sr₂O_{1+x} layers in the newly discovered Sr_{n+1}Cu_nO_{2n+1+x} compounds^{9,10} which are also synthesized at higher pressure, is intriguing. Hiroi *et al.*^{9,10} have speculated that high pressure reduces the mismatch between the

TABLE I Atomic positions for the planar defect compared to those for the non-defective infinite-layer unit cell

Planar defect atomic positions				Corresponding non-defective infinite-layer atomic positions			
Atom	x	y	z	Atom	x	y	z
Cu	0	0	0	Cu	0	0	0
O(1)	0.5	0	0	O	0.5	0	0
O(1)	0	0.5	0	O	0	0.5	0
O(2)*	0	0	0.29	O*	—	—	—
O(2)*	0	0	0.71	O*	—	—	—
				O	0.5	0	0.5
O(3)	0.5	0.5	0.5	O	0	0.5	0.5
Sr(1)*	0	0	0.422	Cu	0	0	0.5
Sr(1)*	0	0	0.578				
Sr(2)	0.5	0.5	0.20	Sr	0.5	0.5	0.25
Sr(2)	0.5	0.5	0.80	Sr	0.5	0.5	0.75

The unit cell for the defect is $3.9 \times 3.9 \times 7.4 \text{ \AA}$ (x, y, z), while that for the non-defective infinite-layer compound is: $3.9 \times 3.9 \times 6.8 \text{ \AA}$ ($x, y, (2)z$). The atomic positions are scaled to the unit-cell length. The staggering of Sr(1) is 0.078 times the unit-cell length along c -axis, which results in Sr(1) positions being, $0.50 - 0.078 = 0.422$ and $0.50 + 0.078 = 0.578$.

* Half occupancy.

† No apical oxygens.

CuO_2 and Sr-O layers, allowing new structures to form. The triple Sr-O layer model can then be thought of as an extension of the double Sr layers present in $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+x}$ compounds. We have not seen any defects with more than three layers in these compounds. While other known hole-doped copper oxide superconductors have a homogeneous occupancy of apical oxygen sites there are no apical oxygen atoms in the non-defective infinite-layer compound. It appears that the planar defects provide at least half of the possible number of the apical oxygens to the CuO_2 planes and therefore permit hole-doped high- T_c superconductivity. (The previously proposed¹⁰ half-occupancy of apical oxygen sites in Sr_2CuO_3 has been called into question by recent neutron-diffraction experiments.¹¹)

It should be pointed out that neither X-ray microanalysis nor HREM imaging can define the oxygen content and locations precisely. The two Sr positions (both Sr(1) and Sr(2)) are determined here with a high degree of confidence, but the O positions are assigned according to the cation positions. Because the cation position is dictated by anion coordination, the observed staggering of Sr(1) is clearly due to the reduction of apical oxygen occupancy, which is also consistent with the charge neutrality arguments.

We speculate that these planar defects can be p-type if doped by excess oxygen ($x > 0$) or n-type with oxygen deficiency ($x < 0$), leading to both p- and n-type superconductivity. Furthermore, for $x = 0$, one can obtain neutral Sr_3O_2 layers. This hypothesis agrees with previous observations of superconductivity in this system⁴ as a function of the synthesis atmosphere, that is p-type with $T_c \approx 110 \text{ K}$ under oxidizing conditions and n-type with $T_c \approx 43 \text{ K}$ under reducing conditions.

We have found that compositions with excess cation ($y > 1$) show an increased Meissner fraction by a factor of almost four, relative to the stoichiometric or $y < 1$ compositions. This is consistent with A-cation-rich planar defects observed in this study. In our most recent experiments on pure SrCuO_2 (that is, no calcium), we also observe planar defects similar to those reported here and they too are Sr-rich. □

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Light-emitting diodes made from cadmium selenide nanocrystals and a semiconducting polymer

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ELECTROLUMINESCENT devices have been developed recently that are based on new materials such as porous silicon¹ and semiconducting polymers^{2,3}. By taking advantage of developments in the preparation and characterization of direct-gap semiconductor nanocrystals^{4–6}, and of electroluminescent polymers⁷, we have now constructed a hybrid organic/inorganic electroluminescent device. Light emission arises from the recombination of holes injected into a layer of semiconducting *p*-paraphenylene vinylene (PPV)^{8–10} with electrons injected into a multilayer film of cadmium selenide nanocrystals. Close matching of the emitting layer of nanocrystals with the work function of the metal contact leads to an operating voltage¹¹ of only 4 V. At low voltages emission from the CdSe layer occurs. Because of the quantum size effect^{19–24} the colour of this emission can be varied from red to yellow by changing the nanocrystal size. At higher voltages green emission from the polymer layer predominates. Thus this device has a degree of voltage tunability of colour.

Nanocrystals normally exist as powders or dissolved in liquids. To employ them in devices it is necessary to develop new techniques of assembly which allow charge injection into, and transport through, nanocrystals. To achieve this goal we construct multilayers of nanocrystals using the well-developed chemistry of thiols on metal surfaces. Narrow size distributions ($\pm 5\%$ of the diameter) of CdSe nanocrystals with absorption maxima in the range 580–620 nm (30–50 Å diameter) are prepared by the TOPO method of Murray and Bawendi⁵ as modified by Bowen Katari et al.⁶. The nanocrystals are dissolved in toluene, and this solution is exposed to either bare indium tin oxide (ITO) or ITO/PPV plates which have been pretreated with hexane dithiol, a bifunctional compound that binds nanocrystals to surfaces^{6,12}. This procedure is repeated five times, leading to the formation of a multilayer structure with closely packed disordered sheets of CdSe nanocrystals separated by organic spacers. This well-characterized buildup process permits the isolated nanocrystals to be investigated before being incorporated into the diode structure. In addition, this process permits nanocrystals with different electronic properties to be placed in defined locations in the same device. The overall thickness of the nanocrystal multilayer is only a few hundred ångströms. This thin layer readily undergoes dielectric breakdown at the voltages required for operation. For this reason, a 1,000-Å-thick layer of PPV, prepared by standard methods (250 °C conversion temperature and absorption maximum at 415 nm), is a desirable

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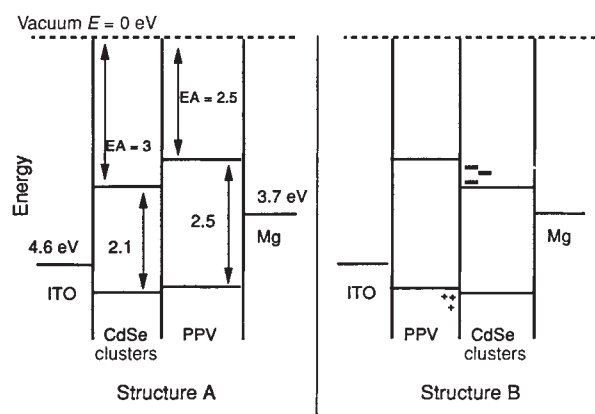


FIG. 1 Energy-level diagram for the composite samples. The work functions of indium tin oxide (ITO) (4.6 eV) and Mg (3.7 eV)²⁵ were provided by the literature; an upper limit for the electron affinity (EA) of PPV (2.5 eV) is assumed¹⁶. The band gaps of the CdSe nanocrystals and PPV are respectively 2.1 eV and 2.5 eV. The electron affinities of different sized CdSe nanocrystals are not known; we estimate a value averaged over many sizes based on the ionization potentials as measured by valence band photoemission²⁶. In structure A the nanocrystals are closest to the ITO and, as can be seen from the Figure, neither the electrons or the holes are confined by the heterojunction. In the opposite arrangement (structure B), the higher electron affinity of CdSe leads to a lower operating voltage, as well as charge confinement at the heterojunction.

component of the sample. It both ensures electrical stability and enhances carrier injection and confinement. Figure 1 shows an energy-level diagram for two geometries, A and B. Electrical contact is made by magnesium coated with silver; X-ray photoemission studies of these contacts indicate that the magnesium is oxidized readily in air.

When subjected to a forward bias, with ITO positively biased with respect to magnesium, both of the sample geometries (Fig. 1) emit with a luminosity of $\sim 100 \text{ cd m}^{-2}$ (visible under normal room lights). In structure A, with PPV closest to the magnesium contact, only green PPV emission is observed. The lack of nanocrystal emission indicates that charge recombination is occurring primarily in the PPV layer. PPV is known to be a poor transport material for electrons, so when it is next to the electron injector in a sandwich configuration the electrons are effectively 'trapped' in the PPV. This puts the recombination zone for electrons and holes in the PPV and close to the metal electrode. A similar phenomenon has been observed in pure polymer bilayer devices that preferentially exhibit emission from the layer closest to the electron injector¹³. The electrical characteristics of structure A samples are quite similar to PPV alone, with operating voltages of $\sim 7 \text{ V}$ and standard diode current-voltage (I/V) behaviour (Fig. 2, top).

Drastically different electro-optic properties are observed if the spatial positions of the nanocrystals and the PPV are reversed, giving structure B (Fig. 2, bottom). Under a forward bias of only 4 V, these samples exhibit an emission characteristic of the nanocrystals. Because the operating voltages of these devices are lower than those of PPV alone, we propose that the electron affinity of the nanocrystals must be greater than that of PPV. Not only does a higher electron affinity decrease the 'turn-on voltage' (that is, the voltage at which charge is injected), but it also leads to a situation in which the electrons traversing the CdSe multilayer are more likely to face a barrier at the PPV/CdSe interface leading to a build-up of negative charge there. As observed for heterojunctions^{13,14} in multilayer polymer structures, confined carriers help the electroluminescent process both by bringing the recombination zone away from the electrodes (where quenching may occur) and by creating large electric fields at the heterojunction which enhance tunnelling of the electrons

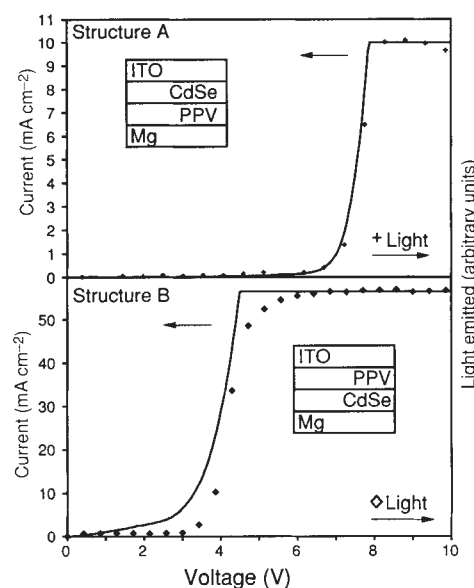


FIG. 2 Electrical characteristics of PPV/CdSe samples. As in plain PPV devices, the current-voltage (I/V) behaviour (solid line) of these devices is similar to that of a classic diode. The barrier to charge injection (and thus the barrier controlling our I/V curves) occurs at a metal/semiconductor junction and thus can be classified as a Schottky barrier. The points (+, ◇) show light intensity versus voltage for structures A and B. In structure A (top), the turn-on voltage is nearly 7 V ($7 \times 10^4 \text{ V cm}^{-1}$), and typical operating currents are 10 mA cm^{-2} . The plateau in the signal is the result of current limiting of the device to avoid shorting, and it is apparent that at this constant current value the emission intensity begins to fall with time under bias. In contrast, the light from structure B (bottom) is relatively stable over time, and the typical operating currents are much higher (50 mA cm^{-2}) with a luminance of 100 cd m^{-2} . Also, the operating voltage is much lower—which is to be expected if the electron affinity of the CdSe multilayer is greater than that of PPV. Under reverse bias, samples experience dielectric breakdown and no electroluminescent light is visible.

into the PPV and holes into the CdSe. The excitons thus formed can diffuse preferentially into the lower-band-gap material giving rise to an emission colour characteristic of recombination in the nanocrystals. Figure 3 clearly shows electroluminescence spectra that shift regularly with nanocrystal diameter (that is, a blue shift with decreasing diameter), thus showing evidence of the quantum confinement effect in nanocrystal electroluminescence.

Whereas most single and bilayer devices fabricated from polymers have been shown to have field dependent I/V characteristics^{11,13} (and therefore tunnelling-based charge injection mechanisms), we find that our samples with varying PPV and CdSe multilayer thicknesses have the same I/V characteristics and therefore proceed by a voltage dependent current injection mechanism. This behaviour suggests that the I/V characteristics are determined by charge (in this case electron) injection at the Mg/CdSe interface rather than the ITO/PPV interface. In addition, the voltage dependence of the I/V characteristics suggests our devices have a different majority carrier injection mechanism as compared to the polymer-only devices. Theories involving thermionic emission or a space charge build-up could explain this voltage dependence. We note that these nanocrystal samples have efficiencies 3–10 times larger than those observed in structure A. External quantum efficiencies for both samples, as well as for samples we prepared from PPV alone, are low (0.001–0.01%). However, given that these diodes are unoptimized, there is no reason to believe that these efficiencies are an upper limit. The behaviour of the samples under reverse bias is difficult to measure because sample breakdown occurs readily in this direction. As in single-layer PPV samples,

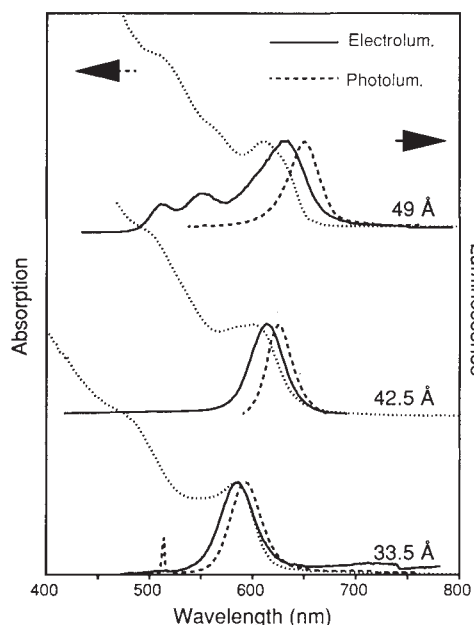


FIG. 3 The quantum confinement effect in the electroluminescence (solid line), photoluminescence (dashed line) and absorption (dotted line) for nanocrystals of three different diameters. The blue shift of the (49, 42.5 and 33.5 Å) electroluminescence with respect to the photoluminescence may be due to trap filling by carriers injected into the CdSe multilayer or to the charge transport mechanism in the CdSe multilayer. The photoluminescence and absorption spectra were taken of nanocrystals dissolved in liquid toluene, whereas the electroluminescence spectra were obtained from our bilayer diode devices. The peak in the photoluminescence spectra at 514 nm is the excitation laser line. The two broad humps (at 515 and 550 nm) in the electroluminescence spectra for the 49 Å sample represent a small amount of emission from the PPV layer, which becomes visible in systems having a thin CdSe layer.

rectification ratios are low and electroluminescence is never observed in reverse bias^{15,16}.

Another interesting aspect of these samples is their voltage-dependent colour (Fig. 4). At lower voltages, emission occurs preferentially in the CdSe layer, whereas at higher voltages the green-coloured PPV layer dominates. Such a phenomenon has never been observed in bilayer structures of organic polymers, even when similar carrier confinement properties are reported. This behaviour is probably a result of the fact that PPV and CdSe nanocrystals are drastically different materials with disparate dielectric constants and transport mechanisms. If the forms of the field dependences of either charge migration or exciton diffusion are different in the two materials, then the recombination zone could spatially move with voltage. It is also possible that the nanocrystals are behaving as typical bulk inorganic diodes, with falling emission intensities at high currents due to heating of the sample¹⁷. Voltage tunability of electroluminescence colour has also been observed in electrochemical cells containing porous silicon¹, but in that case there is a continuous shift in emission colour, the origin of which is not known although it may be related to the presence of silicon 'wires' of different diameter in the sample¹⁸. Our system, in contrast, obtains colour variability from the presence of materials with different emission characteristics. Using this principle, one can envisage creating a multi-colour pixel in which the voltage applied dictates the diode colour. We note that in addition to the voltage dependence of their electroluminescence, the two materials also exhibit different stabilities. If the samples are left under positive bias in air for >10 min, the overall efficiency drops. Although both the PPV and the CdSe peaks shrink with time, the PPV emission disap-

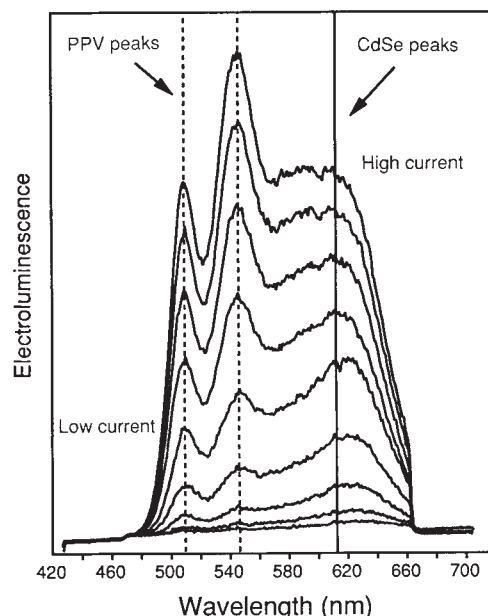


FIG. 4 Voltage-dependent colour of CdSe/PPV samples. In samples with a very thin CdSe layer (one or two monolayers) it is possible to see significant PPV signal in addition to the CdSe signal. At lower voltages CdSe emission dominates, giving a sample with (in this case) a yellow colour. As the voltage is increased the PPV emission becomes more intense, leading to distinctly green luminescence. This phenomenon is completely reproducible over many scans; however, if the sample is kept at bias for >10 min the signal from the PPV will begin to decrease relative to that from the CdSe.

pears altogether, eventually resulting in only nanocrystal emission.

The generation of a light-emitting diode based on an organic/inorganic nanocrystal composite structure built up by assembly of the individual components is a new approach to this technology. Many of the traditional advantages of bulk inorganic semiconductor diodes, namely thermal, chemical and mechanical stability, low operating voltages and high efficiencies can be realized with a material capable of many different emission colours. Moreover, by capitalizing on the established advantages of the organic polymers, such as efficient hole transport and high breakdown voltages, these heterostructures can easily be made large area. Finally, the ability to continuously tune the electron affinity and band gap of the emitting nanocrystal layer by altering the particle size^{19–24} provides a powerful tool for elucidating the role of the heterojunction in the process of electroluminescence. □

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Moisture supply for northern ice-sheet growth during the Last Glacial Maximum

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DURING the last ice age, the Barents Sea ice sheet began to grow 22 kyr ago¹, only 8 kyr before it began to disintegrate². This implies that the ice must have grown very rapidly from the coast to the edge of the continental shelf. Such rapid growth of a large ice sheet requires significant amounts of moisture³, but the origin of this moisture has been unclear, particularly as the CLIMAP climate reconstruction suggests^{4,5} that the Greenland–Iceland–Norwegian (GIN) seas were perennially ice-covered during this period. Here we present data from deep-sea sediment cores from the Fram Strait, which suggest that relatively warm water from the North Atlantic Ocean was advected into the GIN seas in two short-term events (27–22.5 and 19.5–14.5 kyr ago). We suggest that the resulting seasonally ice-free waters were an important regional moisture source for the Barents Sea ice sheet, and that the GIN seas played a much more active role in climate during the last glaciation than has previously been supposed.

Today the Norwegian Sea and the eastern Fram Strait are dominated by the Norwegian current and the Westspitsbergen current which transport warm, Atlantic water from the south to the Arctic Ocean (Fig. 1). This circulation pattern has existed for the past 10 kyr since its re-establishment following the glacial–interglacial transition⁶. In the west, the East Greenland current transports cold, polar water and sea ice from the Arctic Ocean to the south. A comparable circulation pattern probably also existed during the last interglacial (130 kyr ago^{5,7}). A weak influx of Atlantic water to the GIN seas is also recognized during oxygen isotope substage 5a (~77 kyr ago)^{5,7}. In previous reconstructions, isotope stages 4 to 2 were assumed to be characterized by perennial sea-ice cover and by an isolated cold circulation cell in the GIN seas^{4,5} (Fig. 1). More recently, the CLIMAP reconstructions of the Last Glacial Maximum (LGM)⁴ have been challenged by indications of seasonal ice-free conditions in the southern Norwegian Sea^{7–9}.

According to our data from the continental slope and deep sea west of Svalbard (Fig. 1), Atlantic water reached the northern GIN seas during isotope stages 3 and 2. Advection of Atlantic water to the Fram Strait is assumed to have started ~27 kyr ago, as documented in the sediments by high amounts of planktonic foraminifera and coccoliths and, therefore, by a relatively high carbonate content (Fig. 2). Because coccoliths are phytoplankton and therefore require light, the presence of coccoliths in sediments from the Fram Strait and Norwegian Sea is interpreted as an indication of seasonally ice-free waters¹⁰.

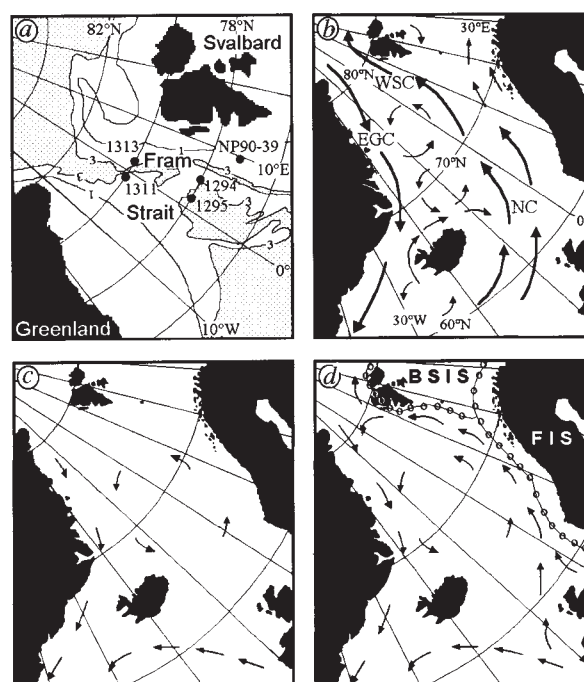
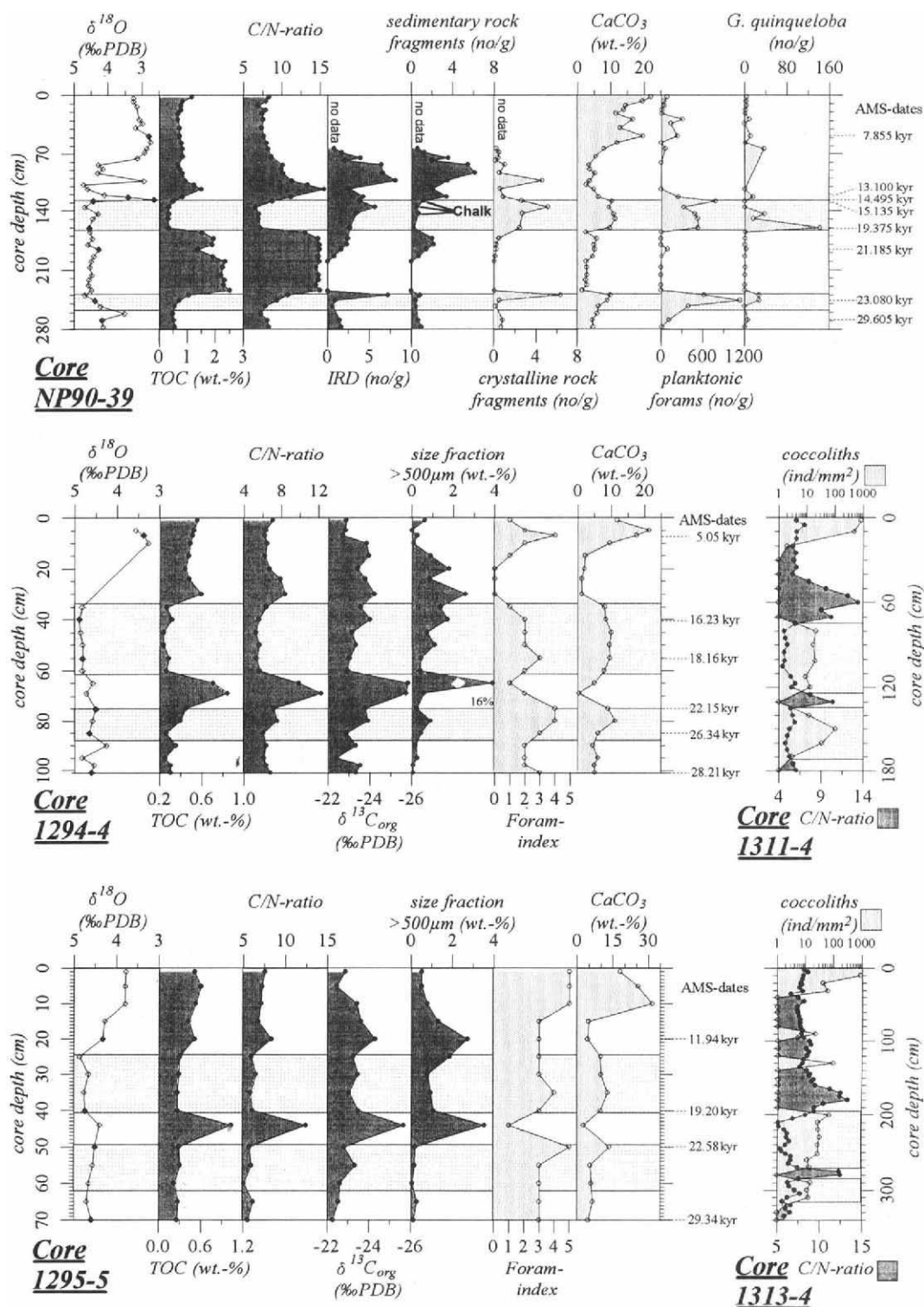


FIG. 1 a, Sites of the investigated cores (NP90–39; 77° 15.5' N, 9° 05.6' E, 2,119 m; 1294–4; 77° 59.9' N, 5° 22.3' E, 2,668 m; 1295–5; 77° 59.2' N, 2° 24.8' E, 3,112 m; 1311–4; 79° 57.8' N, 0° 10.4' E, 2,615 m; 1313–4; 79° 59.9' N, 2° 44.8' E, 2,627 m) and bathymetry of the Fram Strait. 1,000 m and the 3,000 m isobaths are shown with the areas deeper than 3,000 m stippled. b, Present-day surface water oceanography of the Greenland–Iceland–Norwegian seas³⁴. c, Reconstruction of the surface-water circulation for the Last Glacial Maximum (LGM) according to ref. 5. d, Reconstruction of the surface-water circulation for the LGM (19.5–14.5 kyr) and for the period 27–22.5 kyr according to this study. The dotted line indicates the extension of the Barents Sea ice sheet (BSIS) and the Fennoscandian ice sheet (FIS) during the LGM²⁵. (WSC, Westspitsbergen Current; EGC, East Greenland Current, NC, Norwegian Current).

Most glacial sediments in the GIN seas are totally devoid of coccoliths^{10–12}. High numbers of planktonic foraminifera per gram of sediment can be interpreted as reflecting increased surface water productivity and, therefore, point also to seasonally open waters⁵. The species composition of planktonic foraminifera in these high latitudes is clearly dominated by the polar species *Neoglobobulimina pachyderma* (sin.)¹³. In the GIN seas high abundances of the subpolar planktonic foraminifer *Globigerina quinqueloba* are characteristic of the oceanic frontal zones (the polar front, dividing polar and Arctic waters, and the Arctic front, dividing Arctic and Atlantic waters⁹). Therefore, the occurrence of *Globigerina quinqueloba* in core NP9039 (Fig. 2) indicates the advance of the polar front, or possibly the Arctic front, to the Fram Strait during this interval, resulting in at least seasonally open waters.

From the sediment cores it is evident that the open-water conditions are followed by intensified input of terrigenous material. At ~22.5 kyr ago the ice sheet seems to have expanded into the ocean (Fig. 3), where it could directly affect circulation and sedimentation in the GIN seas. Increased input of terrestrial material is evident in several ways. First, the 0.5–1.5% increase in total organic carbon (TOC) content is paralleled by high C/N ratios (here taken to be the ratio of organic carbon (C_{org}) to total nitrogen) and light $\delta^{13}C_{org}$ values (Fig. 2), which are clear indicators of a terrigenous origin for the organic matter¹⁴ as has been shown also for the GIN seas^{15–17}. Second, in core NP90–39 from the Svalbard continental slope (Fig. 1) at ~21 kyr ago, there is a significant increase in ice-rafted detritus (IRD) or

FIG. 2 Sediment data for the investigated cores. The two advection events, when Atlantic water reached the Fram Strait (27–22.5 kyr and 19.5–14.5 kyr), are indicated by stippled bars. For the accelerator mass spectrometry (AMS) ^{14}C dates on cores NP90-39, 1294-4 and 1295-5 ~2,000 specimens of *Neogloboquadrina pachyderma* (*sin.*) were picked. All AMS dates are corrected for ^{13}C and a reservoir age of 440 yr (ref. 35). Stratigraphic interpretation for continental slope cores 1311-4 and 1313-4 is based on previously published data^{11,15} and on graphic correlation to the other three cores. Oxygen isotopes were measured on the planktonic foraminifera *N. pachyderma* (*sin.*). IRD are all terrigenous grains $>500\mu\text{m}$. Numbers of sedimentary and crystalline (including mono-crystalline particles) rock fragments are counted from the same fraction. Countings for planktonic foraminifera and for *Globigerina quinqueloba* were carried out on the $>100\mu\text{m}$ fraction. As other studies from this region have shown, the species composition in this size fraction is more complex, mostly due to a higher relative abundance of *Globigerina quinqueloba*^{9,36,37}, than in the $>150\mu\text{m}$ fraction used by the CLIMAP study^{4,5}. The Foramin-index is a semiquantitative estimate of the content of planktonic foraminifera in the coarse fraction $>63\mu\text{m}$: 0, no forams; 1, 0–5%; 2, 5–20%; 3, 20–50%; 4, 50–80%; and 5, $>80\%$. Coccolith abundances are given in individuals per mm^2 smear slide area and are taken from ref. 11 (note the log scale). All other methods are described elsewhere¹⁵.



dropstones (grains $>500\mu\text{m}$), which are assumed to reflect iceberg transport instead of sea-ice transport¹⁸ (Fig. 2). The input of fine-grained IRD leads that of the coarse-grained components, suggesting that the initial phase of ice advance is associated with a high input of sediments probably supplied by sweeping-out of shelf and fjord sediments onto the continental slope. The amount of coarse-grained material increases ~21 kyr ago, and is dominated by sedimentary rock fragments characteristic of the Svalbard archipelago and the Barents Sea, showing the same high TOC contents and C/N ratios as the fine-grained material (Fig.

2). We suggest that this indicates a stage when ice extension reached a critical level, resulting in increased iceberg production (Fig. 3). Iceberg transport is also reported as an IRD peak in central Fram Strait sediments (cores 1294-4 and 1295-5), again coinciding with high TOC contents and C/N ratios similar to those along the continental slope (Fig. 2).

At ~19.5 kyr ago a second advance of Atlantic water to the Fram Strait is documented in the sediments. Again, this is indicated by high numbers of planktonic foraminifera and the occurrence of coccoliths and *Globigerina quinqueloba* (lower

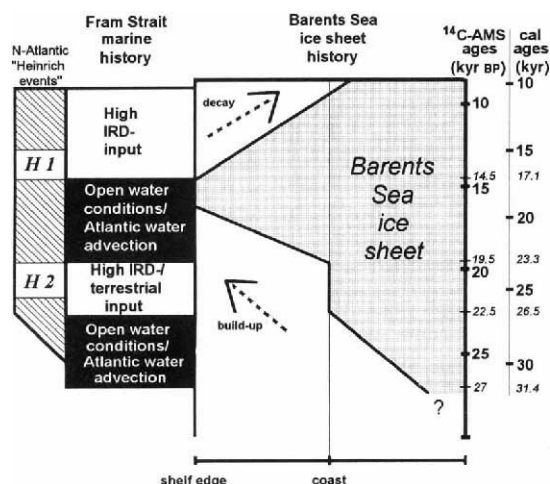


FIG. 3 Reconstruction of the growth and decay of the Late Weichselian Barents Sea ice sheet related to the marine history in the Fram Strait. Ages for the North Atlantic Heinrich events are taken from ref. 31. In addition to the AMS ^{14}C ages (kyr before present, BP) we also give ages calibrated to the U/Th chronology ('cal ages'). For the period 10–20 ^{14}C kyr these calibrated ages are calculated using a linear approximation given by ref. 38. For the period 20–27 ^{14}C kyr we used an extended second-order fit (E. Bard, personal communication). Italicized numbers in the age columns indicate beginnings and endings of the advection events.

numbers in Holocene sediments are due to increased carbonate dissolution, indicated by increased fragmentation along the Svalbard continental slope) (Fig. 2). The CLIMAP reconstructions⁴ indicate closed ice cover for the LGM throughout the GIN seas and even further to the south. CLIMAP reconstructions are based largely on foraminiferal transfer functions¹⁹. The use of these is problematical at high latitudes, due to the mostly mono-specific foraminifera assemblage dominated by the only polar species *Neogloboquadrina pachyderma* (sin.). Therefore, for summer surface water temperatures below $\sim 4^\circ\text{C}$ the results of the transfer-function analyses become inconclusive²⁰.

After 19.5 kyr the IRD content continues to increase, but there is an obvious change to a dominance of crystalline grains, which is also reflected in decreasing TOC contents and C/N ratios, and increasing $\delta^{13}\text{C}_{\text{org}}$ values (Fig. 2). Together with the crystalline fragments we found chalk fragments ($>500\ \mu\text{m}$) in core NP90-39. Similar fragments have also been reported from other cores in the GIN seas associated with both advection events^{18,21,22}. The chalk facies is restricted to the area south of 59°N (ref. 23). Therefore, findings of coarse chalk fragments at 78°N can be explained only by ice-rafting in a northward direction. Because the source areas for the chalk (the North Sea, where chalk fragments have been found in tills²⁴, and the Baltic regions) were glaciated during the Late Weichselian^{25,26}, glacial erosion of chalk rocks followed by ice transport is likely. A northward current, required to transport icebergs from the North Sea area to the Fram Strait, could also have transported icebergs containing crystalline fragments northward from possible source areas along the Norwegian coast^{21,27}, where seasonally open waters are also reported for this period²⁸. Such a current originating far to the south would have been warmer than the polar water of an isolated circulation cell within the GIN seas and would provide seasonal open waters, allowing *Globigerina quinqueloba* and coccoliths to thrive there. We interpret even the small observed coccolith numbers of 10–50 individuals per mm^2 to be indicative of advection because both events are recorded as comparable coccolith peaks in several cores from the GIN seas^{11,12}. Seasonally open water conditions would have led to increased evaporation and precipitation in the area, resulting in further extension of the ice onto the shelf (Fig. 3). Seasonally

open waters in the Fram Strait during the LGM could also be explained by a polynya, but the connection to the North Sea area proved by the chalk fragments excludes this possibility.

During the second Atlantic water advection event (19.5–14.5 kyr ago), the ice expanded to the shelf edge (Fig. 3). Due to rising sea level, however, disintegration of the marine-based, and therefore unstable, ice sheet started shortly after reaching its maximum extension¹. This is documented by (1) a significant shift in IRD provenance (from a crystalline Fennoscandian source to a sedimentary rock source reflecting Svalbard and the Barents Sea) combined with a continuing increase in ice-rafting; (2) an increase in terrestrial organic carbon input (pointing also to a Svalbard/Barents Sea source area); and (3) a strong reduction in carbonate content. The association of these changes with a light $\delta^{18}\text{O}$ peak at 14.5 kyr ago, earlier interpreted as a meltwater signal², supports the idea of early initiation of disintegration of the Barents Sea ice sheet².

These two seasonal open-water events were not necessarily the only moisture source supporting the final build up of the Barents Sea ice sheet. As today, when most of the rainfall over Greenland originates from evaporation in the subtropics²⁹, distant moisture sources are also possible for the LGM. The coincidence between these events and the final ice sheet build-up implies, however, that this regional source was an important contributor, and possibly the decisive trigger, which pushed precipitation beyond the critical level to allow ice-sheet formation.

There is an obvious temporal coincidence between the two phases of increased terrestrial input to the Fram Strait and the two youngest IRD-rich layers in the North Atlantic, called 'Heinrich layers' (H1, ~ 14.5 – 13.5 kyr and H2, ~ 21.4 – 19.9 kyr; refs 30, 31). The carbonate-dominated lithological composition of the IRD in H1 and H2 points to a North American source^{31,32}, whereas in the Fram Strait (and also in contemporaneous diamictons in the Norwegian Sea¹⁷) a Fennoscandian source is implied. Also, in contrast to the Fram Strait events, the Heinrich layers follow a cooling of the surface waters, which might reflect the diversion of temperate Atlantic waters to the GIN seas. Nevertheless, the temporal and sedimentological coincidence between both kinds of events may indicate a common trigger mechanism or a mutual trigger effect. Another Heinrich event, H3, dated in the North Atlantic at 29.2–26.2 kyr (ref. 31) and interpreted to have a different source area from H1 and H2 (ref. 32), is not paralleled by an event in the Fram Strait sediments (Fig. 2). The discovery of possible Heinrich-like events for the Barents Sea ice sheet may indicate a temporary instability of the ice sheet, which was not orbitally forced or caused by sea-level changes. This points to a much more complex relationship between ice dynamics and climate/sea-level changes than previously suggested³³.

The evidence presented here for a meridional circulation pattern and seasonally ice-free waters, combined with indications for deep- and/or intermediate-water ventilation⁸, point to the persistence of thermohaline circulation in the GIN seas during the LGM (Fig. 1). Until now, most climate models for the LGM rely on CLIMAP data and thus on perennially sealed GIN seas. According to our new results, estimates for the LGM of, for example, oceanic pole-ward heat transport, surface albedo, evaporation and deep-water formation in the GIN seas should be recalculated. Climate models considering dynamic GIN seas may indicate that the GIN seas are as important for the LGM global heat budget as they are in our present climate. □

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Decade-scale trans-Pacific propagation and warming effects of an El Niño anomaly

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EL Niño events in the Pacific Ocean can have significant local effects lasting up to two years. For example the 1982–83 El Niño caused increases in the sea-surface height and temperature at the coasts of Ecuador and Peru¹, with important consequences for fish populations^{2,3} and local rainfall⁴. But it has been believed that the long-range effects of El Niño events are restricted to changes transmitted through the atmosphere, for example causing precipitation anomalies over the Sahel⁵. Here we present evidence from modelling and observations that planetary-scale oceanic waves, generated by reflection of equatorial shallow-water waves from the American coasts during the 1982–83 El Niño, have crossed the North Pacific and a decade later caused northward re-routing of the Kuroshio Extension—a strong current that normally advects large amounts of heat from the southern coast of Japan eastwards into the mid-latitude Pacific. This has led to significant increases in sea surface temperature at high latitudes in the northwestern Pacific, of the same amplitude and with the same spatial extent as those seen in the tropics during important El Niño events. These changes may have influenced weather patterns over the North American continent during the past decade, and demonstrate that the oceanic effects of El Niño events can be extremely long-lived.

The observational evidence for this re-routing of a portion of the Kuroshio Extension comes from satellite altimeter and infrared data. Significant changes in sea surface height (SSH) are seen in a combined analysis of altimeter data from the Geosat-Exact Repeat Mission satellite (Geosat-ERM)⁶ and the European Remote Sensing satellite (ERS-1). The resulting change in SSH (Fig. 1a) represents a change in ocean circulation over the interval from 1988 to 1993. Similar results are obtained by a combination of TOPEX/POSEIDON (a joint USA NASA/

FIG. 1 a, Latitude–longitude plot showing change in sea surface height (SSH) from the Geosat-ERM (November 1986 to October 1989) to ERS-1 (April 1992 to March 1993) observations. The colour bar at the bottom of the Figure indicates changes from –15 to 15 cm. The long time period average position of the Kuroshio Extension¹⁷ is plotted so that the positions of the features may be compared. Large-scale circulation in the oceans is approximated by the geostrophic relation, which implies that water flows along lines of constant height with higher SSH to the right when facing downstream. Thus changes in SSH are related to changes in ocean circulation. The high SSH anomaly north of the Kuroshio Extension at 175° E indicates a northward shift of a portion of the Kuroshio Extension's transport. b, Deviation of sea surface temperature (SST) averaged over April 1992 to March 1993 from a mean over 1985–92. Data were supplied by infrared radiometer satellites⁷. The colour bar indicates anomalies from –1 to 1 °C. The high SST anomaly extending eastwards from Japan at ~40° N is just north of the SSH anomaly in a, as would be expected by the circulation changes implied by a. c, Change in model SSH from the time of the Geosat-ERM (November 1986 to October 1989) to the ERS-1 time frame (April 1992 to March 1993). These are the same time periods as covered by the altimeter satellites in a. A ridge of SSH extends from Japan to the Gulf of Alaska. c is provided by a six-layer, 0.25°-resolution, primitive equation ocean model^{18–20} of the global ocean circulation. This model is based on conservation of momentum and mass and has current velocity, ocean pressure and SSH as variables. The boundary follows the 200-m isobath. This simulation is forced only by winds, and is spun up to statistical equilibrium by forcing with the Hellerman and Rosenstein wind stress climatology²¹. Subsequently, the model is forced from 1981 to 1993 with daily 1,000 mb winds derived from the European Centre for Medium-Range Weather Forecasts with the annual mean replaced by that of Hellerman and Rosenstein²⁰. The model reproduces all of the major current systems of the North Pacific including the equatorial currents and the Kuroshio Extension²⁰.

French CNES altimeter satellite) and Geosat-ERM altimeter data.

In Fig. 1a, a positive SSH anomaly centred just south of 40° N at 175° E lies north of the Kuroshio Extension. The anomaly indicates that a portion of the current is re-routed to a path that follows the northern slope of the anomaly. The circulation changes imply changes in sea surface temperature (SST) as warm waters are advected further north. A consistent set of global SSTs has been compiled since 1985 by Reynolds⁷. A warm SST anomaly north of the Kuroshio Extension is clearly seen in Fig. 1b, extending from Japan to North America at 40° N. This SST anomaly is just north of the SSH anomaly as expected (Fig. 1). Thus, both satellite-sensed SSH and SST strongly suggest a northward shift of a portion of the Kuroshio Extension, and a subsequent anomalous northward transport of warm waters.

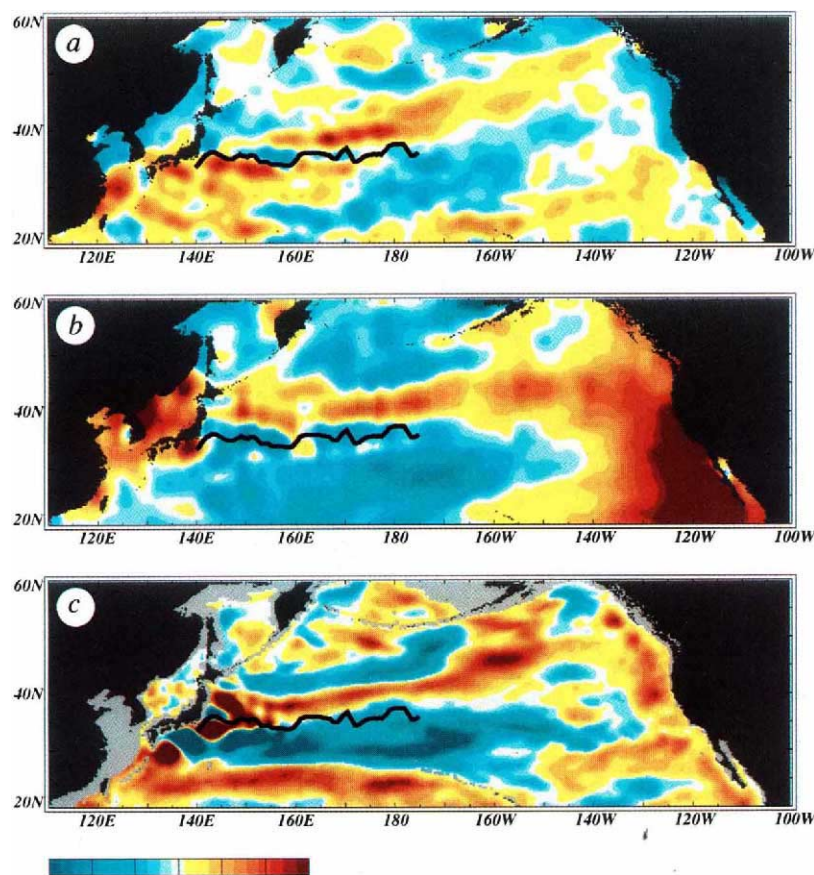
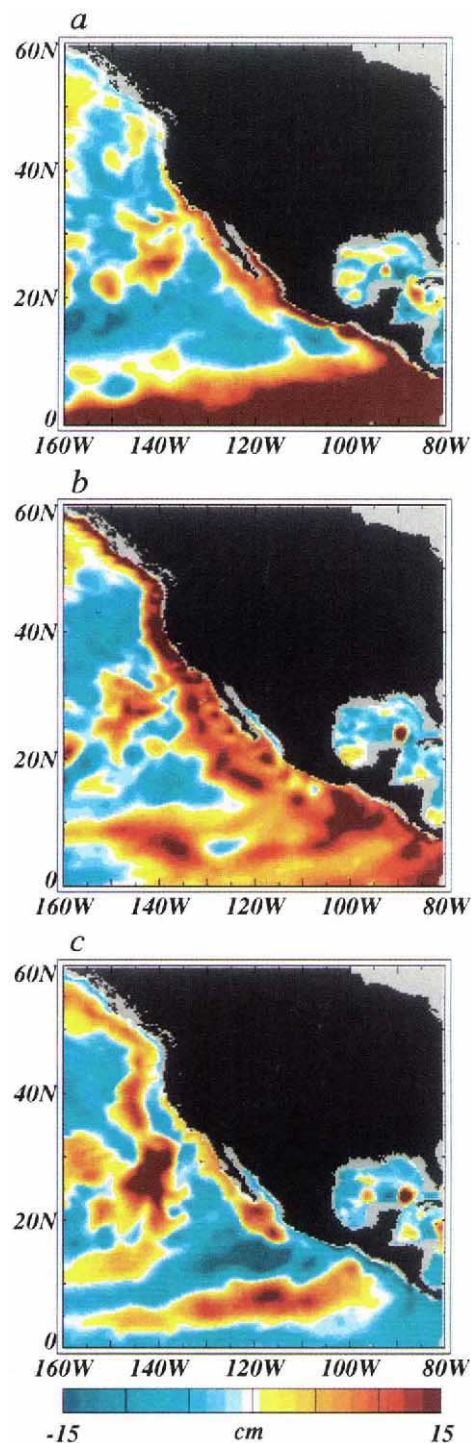


FIG. 2 *a*, Model SSH deviation in August 1982 from a mean over January 1981 to December 1992. The SSH high along the equator and along the coast of the American continents is a signature of the 1982–83 El Niño event, caused by an eastward-propagating equatorial Kelvin wave. The initial equatorial Kelvin wave at the onset of El Niño has been studied analytically²² and numerically^{23,24}. The trans-basin eastward propagation of the equatorial wave requires ~2 months to reach the South American coast, leaving in its wake the positive SSH anomaly extending westwards to the central Pacific. *b*, Model SSH deviation 5 months after the time of *a*. On reaching South America, the equatorial Kelvin wave generates poleward-propagating coastal Kelvin waves along the western coasts of the Americas. The SSH high along the equator diminishes significantly, but coastal Kelvin waves, which propagated up the North American coast, leave a high in SSH along the entire coast. The increase in SSH along the coast, associated with the passage of the coastal Kelvin waves, is visible as far as the Gulf of Alaska. Historically, in response to other El Niño events, coastal Kelvin waves have been observed to propagate along the coast of North America as far as the Aleutian Islands²⁵. *c*, Model SSH deviation 9 months after the time of *a*. The high SSH along the North American continent begins to peel away from the coast as a westward-propagating Rossby wave. (The colour bar at the bottom of the Figure applies to *a*, *b* and *c*).

Concurrent with the recent abundance of satellite data is a revolution in our ability to numerically model the dynamical processes in the oceans. From a numerical model simulation⁸, we use results which cover the 12 years from 1981 to 1993. The model simulation and observational data provide independent results. This synergy is very important because results from one source alone may be inconclusive due to errors or assumptions made. SSH changes in the model simulation (Fig. 1c) indicate an anomaly from Japan to Alaska similar to that of the altimeter data. This suggests that the events that give rise to this ridge are realistically simulated in the model. The decadal simulation provides a unique, continuous, high-resolution data set which is used to identify the sequence of events which generate the

observed trans-Pacific ridge. An animation of the model SSH variations from 1981 to 1993 clearly reveals that these events began in 1982 as a result of the El Niño.

The 1982–83 El Niño was initiated by the relaxation and subsequent reversal of easterly winds over the western and mid-Pacific⁹, which generated an eastward-propagating equatorial Kelvin wave. (A Kelvin wave is a special class of shallow-water waves which occurs along the equator or along coastlines¹⁰.) The Kelvin wave was reflected from the American continents as a westward-propagating Rossby wave, a class of waves that depend on the sphericity and rotation of the Earth (Figs 2 and 3). The Rossby wave reflection process and initial westward propagation in response to the 1982–83 El Niño have been simu-



lated by other ocean models¹¹. The subsequent propagation of the El Niño-generated Rossby wave across the entire North Pacific is summarized in Fig. 3.

The implications of these results are far-reaching and unanticipated. Up to now, propagation of a Rossby wave across the Pacific Ocean at these high latitudes has never been demonstrated. Comparison of the Geosat altimeter data at 30° N with model results verifies the trans-basin journey of the wave (Fig.

4). Rossby waves, observed at the coast of the American continents^{12,13}, were not expected to propagate an appreciable distance into the basin as coherent structures. The energy of the wave was expected to cascade to smaller scales (that is, the wave would dissipate into eddies) and not to propagate as far as Hawaii at this latitude. However, our observations and simulations indicate that the Rossby wave not only remained intact as far as Hawaii, but continued across the basin and is coherent

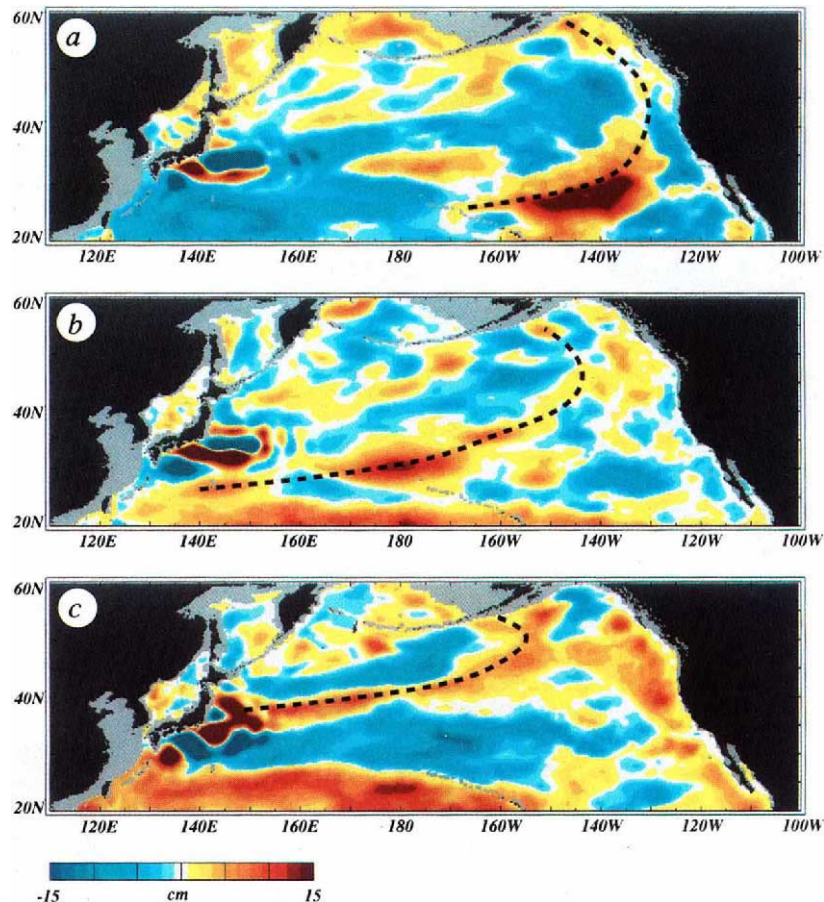


FIG. 3 Model SSH is averaged over 1 year to reduce short-timescale variability (a, over March 1984 to February 1985; b, over May 1987 to April 1988; c, over April 1992 to March 1993), and the 11-year model mean SSH is subtracted. The Rossby wave SSH ridge is indicated by the dashed line. a, Model SSH deviation 2 years after the 1982–83 El Niño. The Rossby wave shown in Fig. 2c has propagated away from the shore. b, Model SSH deviation 5 years after the 1982–83 El Niño. The Rossby wave extends from Taiwan to the Gulf of Alaska. The decrease

in Rossby-wave phase speed with increasing latitude causes bending of the wave front¹². At the northern end, the wave propagates very slowly. Five years after the 1982–83 El Niño, high SSH is still observed near the northwest coast of North America. At the same time, the southern end of the wave has already swept past the Hawaiian Islands. c, Model SSH deviation 10 years after the 1982–83 El Niño. The Rossby wave has moved to a position extending from Japan to Alaska. (The colour bar at the bottom of the Figure applies to a, b and c.)

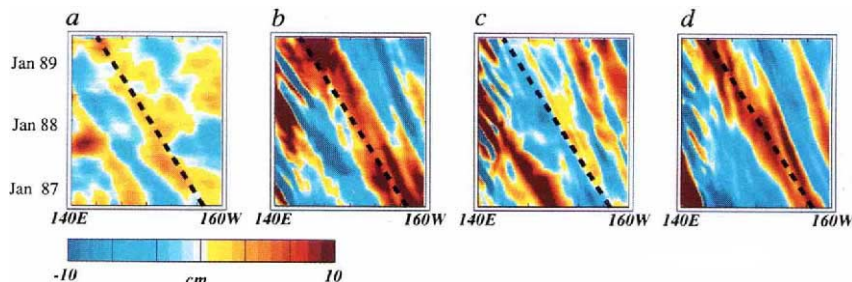


FIG. 4 SSH variations along 30° N from Japan to north of Hawaii; a, from the Geosat altimeter mission and b, from the model over November 1986 to July 1989. The mean SSH from 140° E to 160° W at each point in time has been removed to reduce the effects of the annual steric anomaly due to seasonal heating and cooling of the ocean surface. The dashed line indicates the propagation of the Rossby wave generated by the 1982–83 El Niño across the Pacific Ocean. The propagation speed at 30° N for the wave is 4.9 cm s^{-1} , which is very close to the theoretical value of $\sim 5 \text{ cm s}^{-1}$ for a non-dispersive, internal Rossby

wave. c, In this simulation the wind anomalies which cause the equatorial Kelvin wave associated with the 1982–83 El Niño are removed. The equatorial Kelvin wave and subsequent Rossby wave never occur. d, In this simulation, the predictive power of the model is demonstrated by forcing with observed winds only over the period 1981–84 and winds with only an annual variation afterwards. The equatorial Kelvin wave results, and the Rossby-wave chain of events occurs. This phenomena is therefore predictable on decadal scales. (The colour bar at the bottom of the Figure applies to a, b and c.)

well over a decade later. The Rossby wave generated by the 1982–83 El Niño exists today in the northwest corner of the Pacific Ocean.

Additional evidence for this wave is given by a positive dynamic height anomaly observed in hydrographic data south of Japan at 137° E (ref. 14). This anomaly moves from 18° N in 1984 to almost 30° N in 1989, which agrees well with the movement of the Rossby wave in Figs 3 and 4. Thus, the Rossby wave propagating from North America to the Kuroshio Extension is observed in both the model and the ocean. Two additional ocean model simulations were performed to help isolate the decadal effects of the 1982–83 El Niño. One simulation was initialized in 1984 after the El Niño, and shows the absence of the El Niño-generated Rossby wave at 30° N (Fig. 4c). The other simulation starts in 1981, includes the 1982–83 El Niño, but the wind forcing reverts to an average annual forcing in 1984 to exclude subsequent wind-forced anomalies. It shows the Rossby wave (Fig. 4d), and because no knowledge of actual events in the atmosphere after 1984 was used, it demonstrates the potential for decadal predictions of El Niño-generated Rossby waves and their effects.

In the model, the Rossby wave produces a significant geostrophic velocity anomaly of $\sim 10 \text{ cm s}^{-1}$ in the upper ocean as the wave passes through the Kuroshio Extension in 1991. The geostrophic flow induced by the trailing edge of the Rossby wave reduces the flow of the Kuroshio Extension along 35° N. The leading edge of the Rossby wave induces an eastward velocity perturbation near 40° N. The result is a re-routing of a portion of the Kuroshio Extension transport to $\sim 40^\circ$ N during 1991 and subsequent years. During 1991 the largest SST anomalies are found north of the Kuroshio Extension coincident with the maximum Rossby wave effects on the current. These anomalies reach a peak of $>1^\circ \text{C}$ above mean values observed at other times. After the Rossby wave propagates to the northwest, the geostrophic perturbations on the Kuroshio Extension abate; the extension returns to its normally observed latitude, and the anomalous SSTs begin to disappear.

Corroboration of these events is provided by satellite infrared frontal analyses (J. Szezechowski, unpublished results), which indicate a northward displacement of the Kuroshio Extension in 1990–91 and a southward displacement from 1991 to 1993. In 1992–93, the Rossby wave forms a ridge of high SSH from Japan to Alaska (Fig. 3c). The results presented here indicate that the events giving rise to the trans-Pacific SSH ridge and SST anomaly in 1991–92 originated with the 1982–83 El Niño. Thus the oceanographic effects of a major El Niño have extra-tropical and decadal components.

Climatological effects associated with warm, tropical SST anomalies during major El Niño events are well-documented¹⁵. Surprisingly, the SST anomalies that we observe at much higher latitudes across the North Pacific are of the same amplitude and temporal-spatial extent as those observed in the tropics during major El Niño events. At higher latitudes, significant statistical relationships between SST anomalies in the Pacific Ocean and weather patterns over the North American continent have been found¹⁶. Thus it is possible that the 1982–83 El Niño still has important effects on the climate in 1993. Stated succinctly, the 1982–83 El Niño is not over: its effects have moved from South America to the northwest across the Pacific basin. This Rossby wave should continue to propagate across the far northwest corner of the North Pacific basin for at least another decade, and continue to affect the circulation of the North Pacific.

The deterministic nature of the processes that we report indicates that the ocean is not wholly chaotic and unpredictable, even over basin and decadal scales. Relatively simple planetary wave dynamics, the numerical models that include them, and global satellite data sets provide the necessary tools for long-term oceanic predictions (Fig. 4). Finally, we note that these long-timescale events imply that attempts to determine the climatological or average state of the oceans (a major component

of Earth's climate) will require monitoring of the ocean circulation over decadal timescales. This is indeed a major task which will require synergistic use of numerical ocean models and continuous satellite monitoring for a complete understanding. □

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A unique multitoothed ornithomimosaur dinosaur from the Lower Cretaceous of Spain

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THE Lower Cretaceous lithographic limestones from Las Hoyas (province of Cuenca, Spain) have yielded important vertebrate fossil remains. We report here a new specimen, the first ornithomimosaur theropod found in Europe. *Pelecanimimus polyodon* gen. et sp. nov., has some striking elements preserved, such as the hyoid, sternum and integumentary impressions. The fossil has revealed other unexpected features, including a derived hand in an ancient ornithomimosaur, and a large number of teeth (over 200) with a distinctive morphology. This specimen suggests an alternative evolutionary process towards the toothless condition in Ornithomimosauria, which could be explained by an exaptation. *Pelecanimimus polyodon* stresses the relationship between Troodontidae and Ornithomimosauria.

The Las Hoyas fossil site is one of the most important conservative *lagerstätten* from the Lower Cretaceous of western Europe. It has yielded thousands of specimens including two of the most primitive birds ever found: *Iberomesornis romerali*^{1,2} and *Concornis lacustris*³. A diversified flora and fauna from a lacustrine environment have also been discovered in these lithographic limestones⁴. Non-avian dinosaur remains have not previously been found in Las Hoyas limestones, although some sauropod and *Iguanodon* bones were discovered in equivalent strata of

nearby locations⁵. But in July 1993 remains belonging to a non-avian theropod were discovered.

Theropoda
Tetanurae
Ornithomimosauria
Pelecanimimus polyodon, gen. et sp. nov.

Etymology. *Pelecanus* (Latin), pelican; and *mimus* (Greek), mimic; because of the very long facial part of the skull and the integumentary impressions below the skull, which resemble the gular pouch in the pelican. *Polys* (Greek), many; and *odus* (Greek), teeth; because of its large number of teeth.

Holotype. LH 7777 (Museo de Cuenca, Cuenca, Spain; provisionally housed in the Unidad de Paleontología of the Universidad Autónoma de Madrid, Spain). Articulated anterior half of the skeleton, including skull, complete cervical and almost complete dorsal vertebral series, ribs, pectoral girdle, sternum, right forelimb and nearly complete left forelimb (Fig. 1).

Horizon and locality. Las Hoyas fossil site, Cuenca Province, Spain. Calizas de La Huérguina Formation, Upper Hauterivian–Lower Barremian (Lower Cretaceous)⁶.

Diagnosis. Small ornithomimosaur (2–2.5 m long). Skull with long and shallow snout (maximum length about 4.5 times the maximum height). About 220 teeth: 7 premaxillary, about 30 maxillary and about 75 in the dentary. Heterodont. Maxillary teeth larger than dentary teeth. Teeth unserrated, with a constric-

tion between the crown and the root. There are no complete interdental plates. The maxilla has teeth only in its anterior third, and a sharp edge in the posterior zone. The rostral half of the lower jaw has a straight ventral edge. Ulna and radius are tightly adhered distally. Metacarpal ratio is 0.81:1:0.98.

The skull (Fig. 2a) is nearly complete although the braincase bones are badly crushed. Therefore, the present description is of the external cranial bones from the left half of the skull.

The snout is long and shallow, very similar to *Gallimimus*⁷. As is usual in Ornithomimosauria the premaxilla has a long posteroventral process which has broad contact with the nasal, isolating the maxilla from the external naris. The maxilla presents a long antorbital fossa, which includes one accessory fenestra. The jugal has a long anterior process which contributes to the antorbital fenestra; but the jugal does not reach its rostral border, unlike *Dromiceiomimus*⁸. The dorsal part of the lachrymal forms a small prominence over the level of the prefrontal, which resembles that of *Allosaurus*⁹, but is unlike other ornithomimosaur (although a nasal horn in *Garudimimus* has been reported¹⁰).

The dentition is one of the most striking features of *Pelecanimimus*. Teeth have been previously reported only in *Harpymimus*¹¹, in which 10–12 dentary teeth are present. In *Pelecanimimus* the premaxilla has 7 teeth, the maxilla about 30 and the dentary about 75. It thus has a total of about 220 teeth, the highest tooth count within Theropoda. Unlike any other

FIG. 1 *Pelecanimimus polyodon*. Scale in cm. a, Induced fluorescence ultraviolet light photography. b, Normal light photography. Both pictures were taken before preparation. The slabs that contain the skull and the right hand have been prepared with formic acid after embedding each one in a frame of transparent polyester resin¹⁹ (Fig. 2). The slab that holds the caudal part of the neck, the dorsal vertebrae and the pectoral girdles and humeri is currently being prepared, although some significant features of these bones are reported (see text). c, Coracoids; ha, right hand (see Fig. 2b); hy, hyoid; in, integumentary impressions; lf, left forearm; lh, left humerus; ne, neck; rf, right forearm; rh, right humerus; sc, scapular blade; sk, skull (see Fig. 2a); st, sternum.

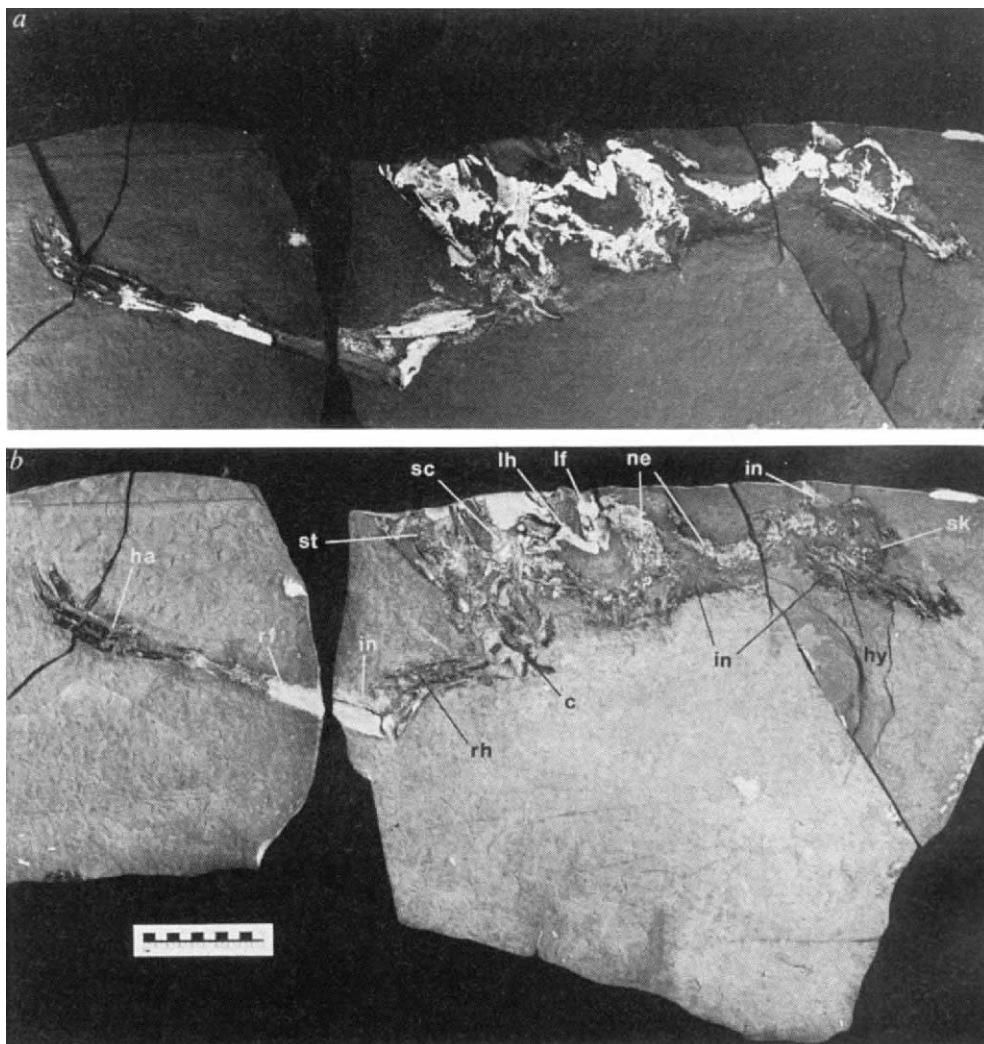
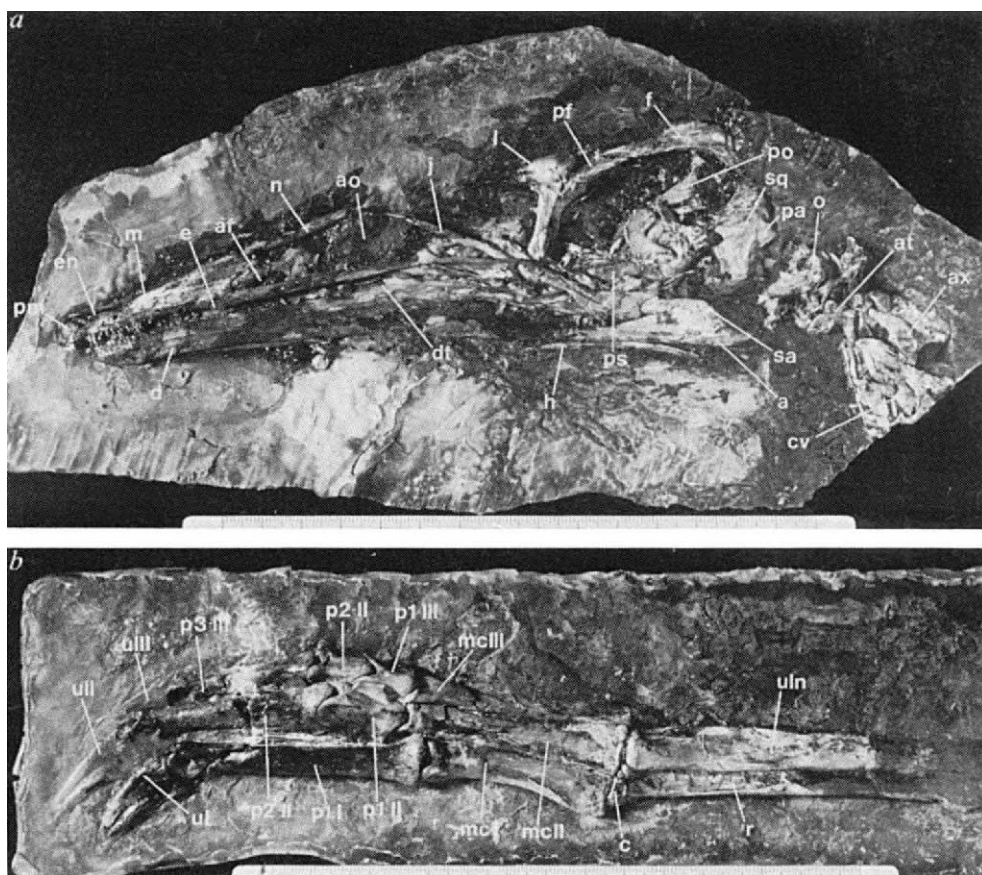


FIG. 2 *Pelecanimimus polyodon*. Scale in mm. a, Left side of the skull, atlas, axis and incomplete third cervical vertebra. The facial half of the skull is almost perfectly preserved, but the posterior half is badly crushed. The occipital part of the skull is disconnected from the rest. Therefore, until the counterslab is prepared to allow for a detailed comparison, the identification of some of the bones of this zone remains tentative. a, Angular; af, accessory fenestra; ao, antorbital fenestra; at, atlas; ax, axis; cv, third cervical vertebra; d, dentary; dt, dentary tooth (the most posterior is marked); e, sharp edge of the maxilla; en, external naris; f, frontal; h, hyoid; j, jugal; m, maxilla; n, nasal; o, occipital part of the skull; pa, parietal; pf, prefrontal; pm, premaxilla; po, postorbital; ps, bulbous parasphenoid capsule; sa, surangular; sq, squamosal. b, Extensor side of the right hand and distal part of the forearm. The unguals of the second and third digits and parts of the Mc III are preserved in the counterslab, although they have been copied by the polyester resin in the slab shown. The proximal zone of the forearm (ulna and radius) has been dissolved, but has also been copied by the resin. c, Carpals; mcl, first metacarpal; mcll, second metacarpal; mclll, third metacarpal; p1-I, first phalanx of first digit; p1-II, first phalanx of second digit; p2-II, second phalanx of second digit; p1-III, first phalanx of the third digit; p2-III, second phalanx of third digit; p3-III,



third phalanx of third digit; r, radius; uln, ulna; ul, ungual of the first digit; ull, ungual of the second digit; ulll, ungual of the third digit.

theropod, the maxillary teeth are located only in the anterior third of the maxilla, with a sharp edge in the posterior zone. Also surprising is the morphology of the teeth, which are similar to those of Troodontidae¹² (including *Sinornithoides*¹³, from the Lower Cretaceous of China): the teeth have a basal constriction in the crown, and there are no interdental plates. Unlike troodontids, the teeth are unserrated. The anterior teeth have enamel; a more detailed analysis of the posterior ones will be necessary. The premaxillary tooth crowns are incisiform and D-shaped in cross-section. The anterior maxillary teeth are similar to the premaxillary ones, and they gradually become blade-like towards the posterior zone, with mesial and distal carinae. The dentary teeth are clearly smaller than those of the upper jaw, as in troodontids¹². The anterior dentary teeth have bulky crowns, which posteriorly become smaller and more slender.

The hyoid apparatus is rarely preserved in dinosaurs, and *Pelecanimimus* preserves the first hyoid known among Ornithomimosauria. It is V-shaped, with its apex rostrally directed, and is one-third of the skull length.

As is usual in ornithomimosaur, the presacral vertebrae seem to lack pleurocoels¹⁴, and have platycoelous centra. The neck length is about twice the skull length. The cervical ribs are not fused to the centra, as in *Gallimimus*⁷.

The sternum is not usually preserved in theropods (this has been interpreted either as a preservational bias or as an unossified structure) and was unknown in Ornithomimosauria. Nevertheless, its presence was suspected because of the great distance between coracoids observed in some cases¹⁵. *Pelecanimimus* has a large (and probably paired) sternum, including a rib margin.

As is usual in ornithomimosaur¹⁵, the coracoid has a very conspicuous biceps tubercle and a large posterior process, and

the humerus has a straight shaft. The ulna and radius are tightly adhered distally (Fig. 2b), which could even be a syndesmosis, as in *Struthiomimus altus*¹⁵.

There are five carpals, as in *Struthiomimus*¹⁵. The three metacarpals are close together for nearly all their length. The ratio McI : McII : McIII is 0.81 : 1 : 0.98. The interphalangeal articulations are ginglymous, although articulations with the metacarpals are simpler. Phalanx 1-I is the longest bone of the hand. The length of the 3-III is greater than the combined lengths of phalanges 1-III and 2-III. Unguals are slightly curved, and the flexor tubercles are poorly developed and distally placed. The three digits are subequal in length (as in most derived ornithomimosaur¹⁴) and almost parallel (unlike *Struthiomimus altus*¹⁵, in which the first digit is oriented towards the second and third during flexion). In *Pelecanimimus*, the hand could have worked like a hook, as in *Gallimimus* or *Ornithomimus*¹⁴.

Another surprising feature of *Pelecanimimus* is the preserved impressions that can be clearly observed below the skull, running below the neck and around the right humerus and elbow (Fig. 1). These probably correspond to integumentary structures. They are composed of a primary system of subparallel fibres arranged perpendicular to the bone surface, and a less conspicuous secondary system parallel to it. There is another impression that might correspond to a soft occipital crest. These impressions are the main reason why the counterslabs have not been prepared so far.

The phylogenetic hypothesis (Fig. 3) supports an unexpected approach, involving exaptation¹⁶, which might explain the evolutionary process towards the toothless condition in Ornithomimosauria. Until now, a progressive reduction in the number of teeth has been considered as the most likely explanation¹⁴: the

<i>a</i>	1				22
<i>Allosaurus</i>	00000	00000	00000	00000	00
<i>Albertosaurus</i>	00000	00010	00001	0010-	-0
<i>Deinonychus</i>	00000	00010	00100	00001	00
<i>Garudimimus</i>	10---	11100	1----	-----	--
<i>Gallimimus</i>	10---	11111	11111	1-111	11
<i>Pelecanimimus</i>	01111	10110	11111	11111	11
Troodontidae	01111	11010	10-10	10001	00

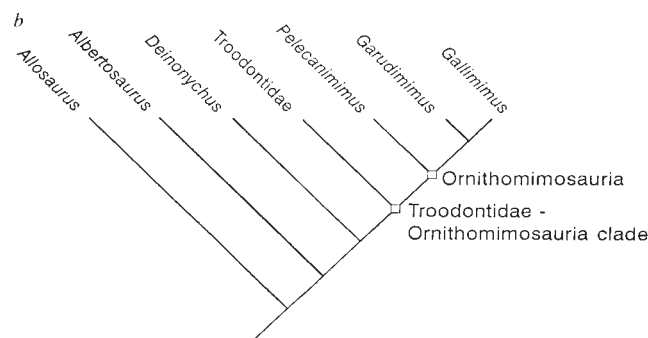


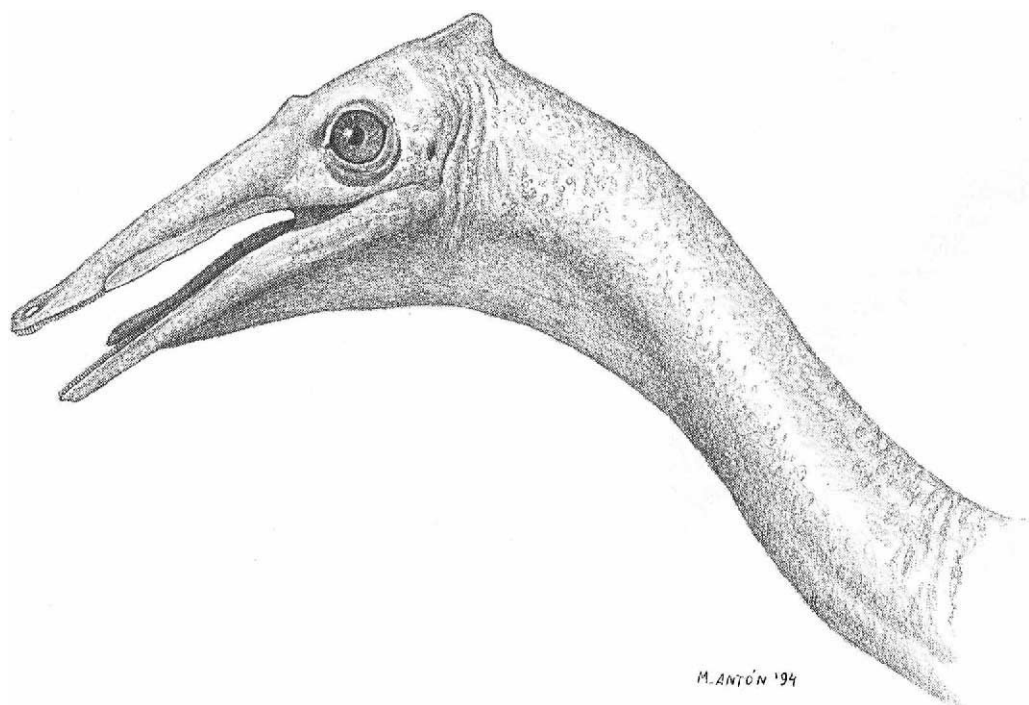
FIG. 3 Phylogenetic analysis. *a*, Matrix of taxa versus characters. Character states: 0, plesiomorphic state; 1, apomorphic state; -, not preserved/unknown. Derived character states: 1, absence of teeth. The absence of teeth is synapomorphic for all the ornithomimosaurs except *Harpymimus*¹¹ and *Pelecanimimus*. 2, Number of teeth higher than 100. *Allosaurus* has a total of 64–78 teeth⁹, *Deinonychus*, 70 and *Albertosaurus*, 62. Troodontidae have a maximum of 120 teeth¹², and *Pelecanimimus*, has about 220 teeth. 3, Maxillary teeth larger than dentary teeth. 4, Basal constriction in the crown of the teeth. 5, Absence of interdental plates. 6, Skull narrow and shallow, with elongated facial part. 7, Orbit length greater than the antorbital fenestra length. 8, Broad contact between the premaxilla and the nasal. 9, Ventral border of the antorbital fenestra is formed by the maxilla and jugal. 10, Rostral half of the lower jaw ventrally concave. 11, Presence of parasphenoid bulbous capsule. 12, Neck length at least twice the skull length. 13, Coracoid biceps tubercle conspicuous and well developed. 14, Posterior

coracoid process conspicuous and well developed. 15, Humeral shaft straight. 16, Hand/humerus+radius length ratio less than 66%. 17, Ulnar and radial distal ends closely joined, even with a syndesmosis. This character state is unknown in *Gallimimus*⁷, but derived in *Harpymimus*¹¹ and *Struthiomimus*¹⁵. 18, Carpals flat, discoidal. 19, Length of mcl greater than half the length of mcll. 20, Length of the phalanx p3-III greater than that of phalanges p1-III+p2-III. 21, First phalanx of digit I of the hand greater in length than Mcll. 22, Flexor tubercle of unguals not well developed and distally placed. *b*, Phylogenetic hypothesis. The data matrix was analysed using the implicit enumeration option of ver. 1.5 of the 'Hennig 86' program²². The result is a tree with a consistency index of 0.81 and 28 evolutionary steps. The Troodontidae–Ornithomimosauria clade (previously discussed²³) is defined by seven synapomorphies: characters 3, 4, 5, 6, 11, 14 and 16. *Pelecanimimus* shares seven derived character states with the remaining ornithomimosaurs: 8, 12, 15, 18, 19, 21 and 22.

primitive tetanurine theropods have up to 80 teeth with tall blade-like crowns, and the primitive ornithomimosaurs have only a few small teeth. The phylogenetic hypothesis suggests an alternative evolutionary process based on a functional analysis of increasing numbers of teeth. A high number of teeth with enough interdental space and properly placed denticles (as in troodontids) would be an adaptation for cutting and ripping. On the other hand, an excessive number of teeth with no interdental space (as in *Pelecanimimus*) would be a functional counterpart of the cutting edge of a beak. Thus, increasing the

number of teeth would be an adaptation for cutting and ripping, as long as the space between adjacent teeth was preserved (Troodontidae–Ornithomimosauria clade, Fig. 3*b*), while it would have the effect of working as a beak if spaces were filled by more teeth (node Ornithomimosauria, Fig. 3*b*). The adaptation to a cut-and-rip function therefore becomes an exaptation¹⁶ with a slicing effect, eventually leading to the cutting edge seen in most ornithomimosaurs. This tendency is shown by the posterior–anterior replacement of teeth by a cutting border on the maxilla of *Pelecanimimus*.

FIG. 4 Hypothetical reconstruction of *Pelecanimimus* based on cranial and vertebral remains.



The present-day ornithomimosaur European record^{17,18} is doubtful; *Pelecanimimus polyodon* is the first unambiguous evidence of these theropod dinosaurs in Europe. Recent findings seem to indicate that Ornithomimosauria is a more widely distributed group (in space and time) than previously accepted. The reason why the present-day known record is essentially Upper Cretaceous remains unclear. □

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Active anaphylaxis in IgE-deficient mice

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THE IgE-triggered release of mast cell mediators in response to antigen is thought to be the primary event in immediate hypersensitivity reactions such as systemic anaphylaxis¹. Although mast cells and basophils can be activated *in vitro* by non-IgE stimuli^{2–5}, it is not known whether these triggers lead to physiological changes *in vivo*. To investigate this possibility, we generated mice with a homozygous null mutation of the *Cε* gene. Such mice make no IgE, but produce other immunoglobulin isotypes normally. We report that despite the IgE deficiency, sensitized mutant mice become anaphylactic on antigen challenge and display tachycardia and pulmonary function changes similar to those seen in wild-type animals. These responses are accompanied by vascular leak, sharply elevated plasma histamine and rapid death. IgE-independent anaphylaxis does not depend on complement activation, but, as indicated in studies using genetically immunodeficient *RAG-2*[–]

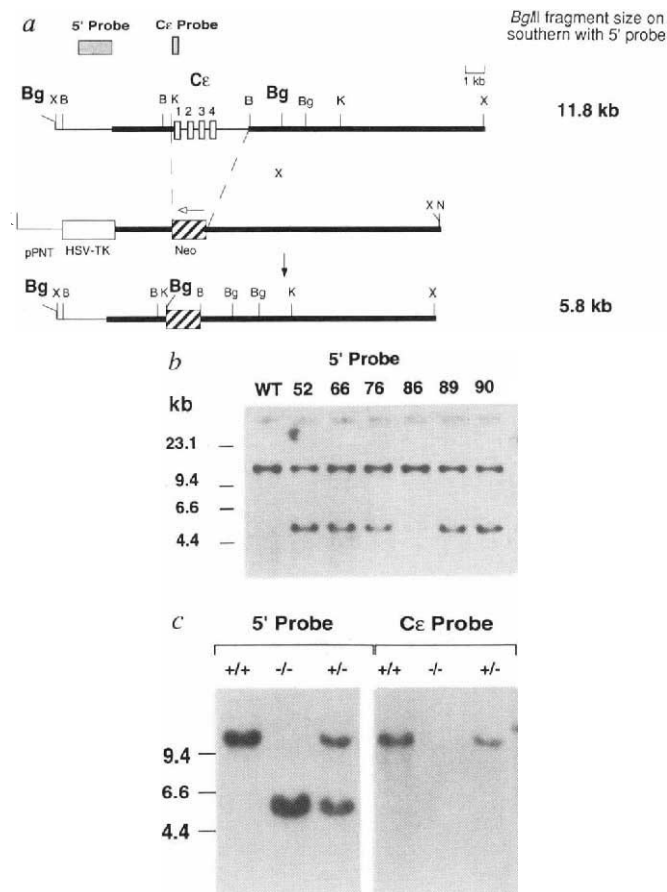


FIG. 1 Generation of mice with complete deletion of the *Cε* exons. **a**, The targeting vector. The replacement-type targeting construct contained 3.2 kb of genomic DNA upstream of the *Cε* exons and 10 kb of downstream homologous sequence (thick lines). The positions of a neomycin resistance gene (Neo, hatched box) replacing the *Cε* exons as well as a herpes simplex virus thymidine kinase gene (HSV-TK, white box) for positive/negative selection are indicated. *Bgl*III sites giving rise to a restriction fragment length polymorphism between the wild-type locus and the targeted allele using a 5' flanking probe are shown (Bg, *Bgl*III; X, *Xho*I; B, *Bam*HI; K, *Kpn*I). **b**, Identification of targeted ES clones. *Bgl*III-digested genomic DNA from ES clones was analysed by Southern blotting using a 5' flanking probe. A 5.8 kb band diagnostic of homologous recombination was present in the six clones (of 100 screened) shown. The first lane contains wild-type DNA. **c**, Southern analysis of tail DNA from the offspring of chimaeric mice generated by blastocyst injection with clone-89 ES cells. Three genotypes, +/+, –/– and +/- were detected. Rehybridization of the same DNAs with a *Cε* exon-specific probe confirmed that this sequence was absent from the genomic DNA of –/– animals.

METHODS. 129/Sv genomic clones containing the *Cε* exons and flanking sequences were isolated from a λ FIX II library (Stratagene) using a *Cε*1 probe generated by PCR. The vector pPNT, kindly provided by V. Tybulewicz, was used to prepare the targeting construct²⁷. pPNT contains the neomycin resistance gene (neo) under the control of the phosphoglycerate kinase promoter (PGK). The linearized targeting construct (25 μ g) was introduced into 10^7 J1 ES cells (kindly provided by En Li and R. Jaenisch) by electroporation²⁸. Selection was initiated at 24 h using G418 and the gancyclovir analogue, FIAU. Clones were recovered at 8 days, aliquots frozen and DNA prepared for Southern analysis using the 5' flanking probe. Clone-89 was injected into blastocysts and the resultant chimaeric male pups mated with black Swiss females (Taconic). The offspring of these matings included animals heterozygous for the *Cε* disruption. These were then bred to homozygosity.

and *SCID* mice, does require a functional immune system. Such results clearly demonstrate that non-IgE pathways for hypersensitivity reactions exist in mice.

A null mutation in the IgE gene was prepared by deleting exons *Cε*1–4 encoding the constant region domains of the ϵ -

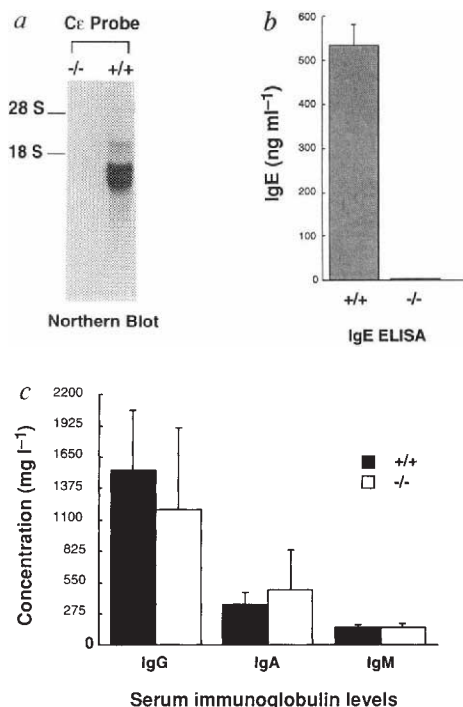


FIG. 2 Absence of IgE mRNA and protein in homozygous mutants; presence of normal IgG, IgA and IgM. *a*, Northern blot of mRNA from IL-4/LPS-stimulated wild-type (+/+) and mutant (-/-) splenocytes using a C ϵ 1 probe. *b*, IgE levels in supernatants of the same cultures. *c*, Serum immunoglobulin levels in 12-week-old wild-type (black bars) and C ϵ mutant (white bars) mice.

METHODS. Single-cell suspensions were prepared from the spleens of wild-type and mutant mice. Following erythrocyte lysis with NH₄Cl, these were cultured at a starting density of 10^6 cells per ml in the presence of IL-4 at 5 ng ml^{-1} (Peprotek) and LPS $25 \mu\text{g ml}^{-1}$ (Sigma)²⁹. Total cellular RNA was prepared on day 5 from 5×10^7 cells using RNazol B (Biotech). This was separated in a 0.75% formaldehyde gel, transferred to Gene Screen nylon membrane and hybridized with a C ϵ 1 specific probe (see Fig. 1*a*). Supernatants from the same 5-day cultures were applied to 96-well plates which had been precoated with polyclonal sheep anti-mouse IgE at 1/500 dilution (The Binding Site) and blocked with 10% rat serum. After an overnight incubation at 4 °C, plates were washed with PBS/0.05% Tween. A biotinylated monoclonal rat anti-mouse IgE (Pharmingen) was then added at $4 \mu\text{g ml}^{-1}$. Plates were developed with horseradish peroxidase streptavidin at 1/1,000 and ABTS substrate (Zymed) and analysed on an ELISA reader at 405 nm. Standard curves were constructed using mouse IgE (Pharmingen) and the IgE concentration of samples calculated ($\text{ng ml}^{-1} \pm \text{s.d.}$). The limit of detection of this assay was $\sim 10 \text{ ng ml}^{-1}$ IgE. Serum was obtained from three 12-week-old IgE-deficient and littermate wild-type control animals. IgG, IgA and IgM levels ($\text{mg l}^{-1} \pm \text{s.d.}$) were determined by radial immunodiffusion using a commercially available kit (The Binding Site).

heavy chain of IgE^{6,7} using a replacement-type targeting construct (Fig. 1*a*). Of 100 transfected embryonic stem (ES) cell clones, 6 underwent homologous recombination (Fig. 1*b*). Chimaeras generated by blastocyst injection with clone ϵ -89 cells were used to breed mice with the targeted mutation (Fig. 1*c*). Homozygous mutants had no genomic DNA hybridizing with a C ϵ 1-specific probe, consistent with the expected deletion of all the C ϵ exons.

IgE is not normally present in mouse serum at significant levels. To confirm that we had generated a null mutation for IgE, we determined cellular messenger RNA and secreted IgE in splenocytes cultured in the presence of interleukin-4 (IL-4) and lipopolysaccharide (LPS), potent inducers of IgE synthesis⁸. Normal splenocytes contained abundant C ϵ transcripts (Fig. 2*a*). No ϵ -mRNA was present in identically stimulated cells from

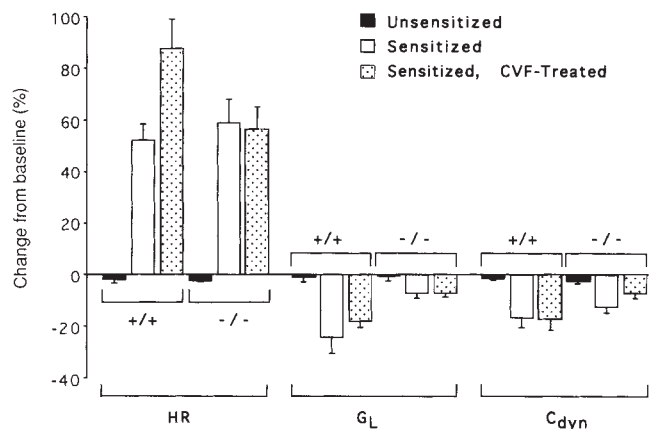


FIG. 3 Cardiac and pulmonary responses of wild-type and IgE-deficient mice undergoing active anaphylaxis to ovalbumin. Heart rate (HR), pulmonary conductance (G_L) and pulmonary dynamic compliance (C_{dyn}) of unsensitized (black bars), OVA-immunized (white bars) and immunized, cobra venom factor-treated (stippled bars) mice 3 min after i.v. OVA challenge. Physiological parameters are represented as mean per cent change from baseline $\pm$ s.e.m. (4–7 mice per group).

METHODS. Wild-type and IgE-deficient littermates were given i.p. injections of OVA (100 μg), alum (1 mg) and 300 ng pertussis toxin (List Biochemicals). Sham immunizations with alum and pertussis toxin were given to 'unsensitized' animals. 18–21 days after immunization, the mice were anaesthetized, tracheotomized and mechanically ventilated in a plethysmographic system for monitoring HR, G_L and C_{dyn} as previously described¹³. Following the determination of baseline values for each physiological parameter, the mice were injected with 500 μg OVA i.v. and monitored for 40 min. Responses were maximal at 3 min for each parameter. Some sensitized animals were depleted of complement by injection i.p. of 10 μg cobra venom factor (Calbiochem) 24 h before antigen challenge. Serum taken from identically treated littermates had no remaining complement activity as measured in a standard haemolysis assay using rabbit red blood cells sensitized with goat anti-rabbit RBC serum³⁰. The responses of the sensitized mice (with or without cobra venom factor treatment) were significantly different ($P < 0.026$) from those of the unsensitized mice for each parameter.

homozygous mutant animals. The wild-type splenocytes secreted large amounts of IgE, but none was produced by mutant cells (Fig. 2*b*). Disruption of the C ϵ locus did not appear to interfere with the production of other immunoglobulin isotypes. Serum IgG, IgA and IgM levels were normal in IgE-deficient mice (Fig. 2*c*).

Systemic anaphylaxis is a rapid, often fatal, immunological reaction characterized by bronchospasm, tachycardia, vascular leakage and hypotension. In rodents, immunization using aluminium hydroxide (alum) and pertussis toxin as adjuvant has been shown to induce production of specific IgE with anaphylaxis resulting on intravenous antigen challenge^{9–12}. We immunized wild-type and IgE-deficient mice with ovalbumin (OVA), alum and pertussis toxin. Animals were challenged by intravenous administration of OVA after 18–21 days. To obtain as much physiological information as possible, the mice were anaesthetized, tracheotomized and monitored in a whole-body plethysmograph for 40 min.

All wild-type mice displayed a rapid increase in heart rate (HR) along with noticeable decreases in pulmonary conductance (G_L) and pulmonary dynamic compliance (C_{dyn}) (Fig. 3). These changes were evident 1 min after antigen administration and became maximal at 3 min. The animals died within 20 min (6/6 deaths). Unsensitized mice, challenged in the same way, displayed no noticeable physiological changes over the course of the experiments (0/7 deaths). OVA-sensitized IgE-deficient animals had the same dramatic responses as normal mice (6/7 deaths). Intravenous infusion of an anti-IgE antibody capable of activating mast cells and basophils by IgE-crosslinking induced the same set of physiological changes in wild-type mice, suggesting

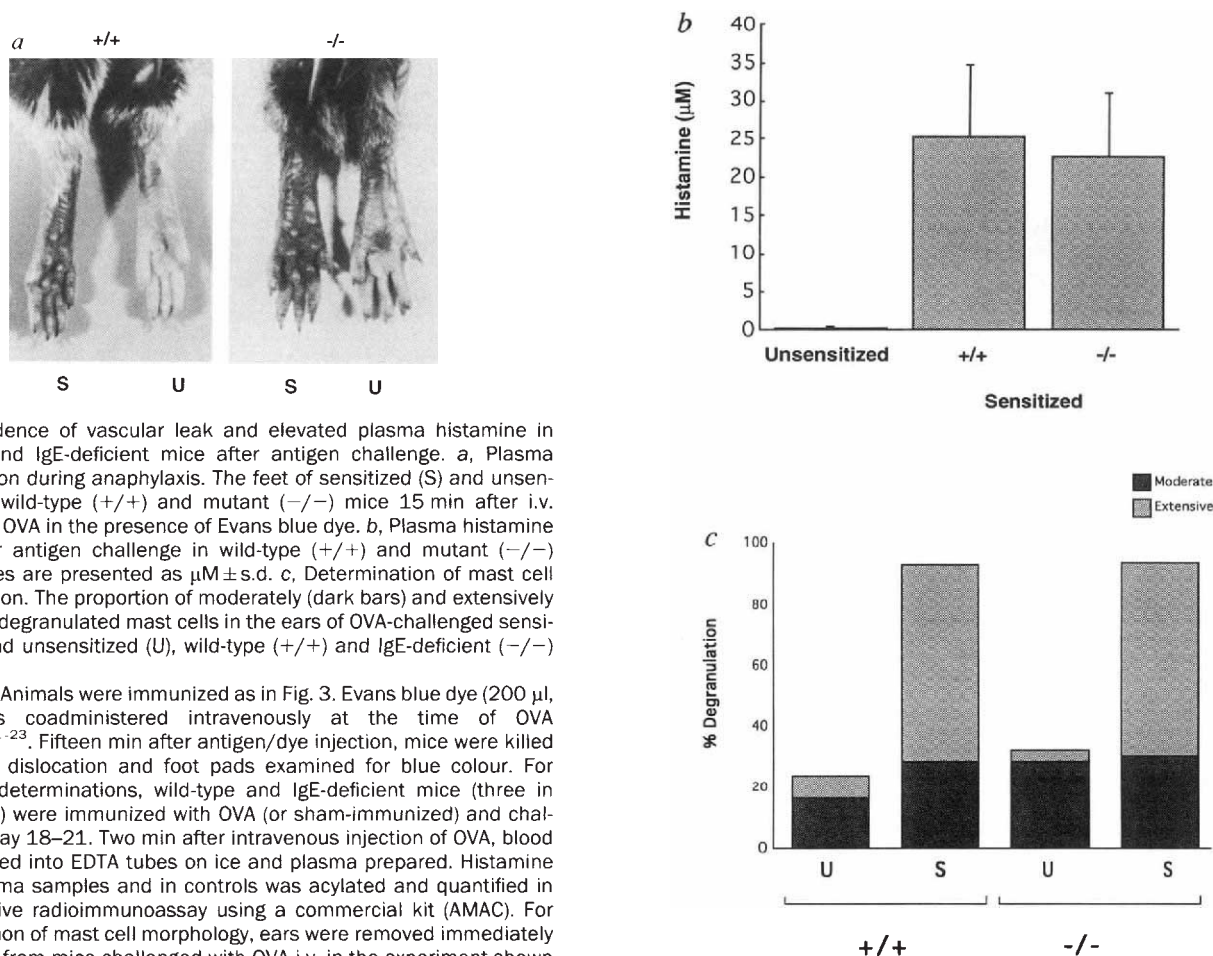


FIG. 4 Evidence of vascular leak and elevated plasma histamine in wild-type and IgE-deficient mice after antigen challenge. **a**, Plasma extravasation during anaphylaxis. The feet of sensitized (S) and unsensitized (U) wild-type (+/+) and mutant (-/-) mice 15 min after i.v. injection of OVA in the presence of Evans blue dye. **b**, Plasma histamine 2 min after antigen challenge in wild-type (+/+) and mutant (-/-) mice. Values are presented as $\mu\text{M} \pm \text{s.d.}$ **c**, Determination of mast cell degranulation. The proportion of moderately (dark bars) and extensively (light bars) degranulated mast cells in the ears of OVA-challenged sensitized (S) and unsensitized (U), wild-type (+/+) and IgE-deficient (-/-) mice.

METHODS. Animals were immunized as in Fig. 3. Evans blue dye (200 μl , 0.5%) was coadministered intravenously at the time of OVA challenge²¹⁻²³. Fifteen min after antigen/dye injection, mice were killed by cervical dislocation and foot pads examined for blue colour. For histamine determinations, wild-type and IgE-deficient mice (three in each group) were immunized with OVA (or sham-immunized) and challenged at day 18-21. Two min after intravenous injection of OVA, blood was collected into EDTA tubes on ice and plasma prepared. Histamine in the plasma samples and in controls was acylated and quantified in a competitive radioimmunoassay using a commercial kit (AMAC). For determination of mast cell morphology, ears were removed immediately after death from mice challenged with OVA i.v. in the experiment shown in Fig. 3. After fixation as previously described¹³, the tissue was processed into 3 μm paraffin-embedded, Giemsa-stained sections. These were examined at $\times 400$ by light microscopy by an observer unaware of their identity. Mast cells were classified as 'extensively

degranulated' (>50% of the cytoplasmic granules exhibiting fusion, staining alterations or extrusion from the cell), 'moderately degranulated' (10-50% of the granules exhibiting fusion or discharge) or 'normal'. Ears were examined from 3 to 5 animals in each group.

that mast cell activation accounted for the responses observed after antigen challenge (data not shown). As expected, anti-IgE had no effect on the mutant mice.

The physiological changes we observed are consistent with those previously reported in murine models of active anaphylaxis^{13,14}. The tachycardia probably results from direct cardiac stimulation by mast cell mediators. The decreased G_L is suggestive of airway obstruction from bronchospasm or mucosal oedema, whereas the drop in C_{dyn} is consistent with the presence of pulmonary interstitial oedema. Although the increase in HR and the fall in C_{dyn} appeared to be similar in wild-type and IgE-deficient mice, the drop in G_L was not as pronounced in the mutant animals suggesting that less airways obstruction occurs in the absence of IgE.

As the anaphylatoxins C3a and C5a are potent mast cell activators *in vitro*⁵, we attempted to induce anaphylaxis in sensitized animals completely depleted of complement using cobra venom factor (CVF)¹⁵. These mice responded to OVA challenge in the same fashion as untreated animals (Fig. 3), indicating that IgE-independent systemic anaphylaxis does not require the generation of complement fragments.

In addition to eliciting anti-OVA IgE in wild-type mice, the immunization protocol we used induced anti-OVA IgM, IgG1 and IgG2a in both wild-type and mutant animals as determined by an isotype-specific enzyme-linked immunosorbent assay (ELISA)¹⁶. It is possible that the OVA-specific IgG stimulated mast cells directly through their Fc γ receptors, a phenomenon previously reported for cultured mast cells²⁻⁴. IgG can also transfer cutaneous hypersensitivity in a model known as passive cut-

aneous anaphylaxis^{17,18}. Immunodeficient animals of the *SCID*/*SCID*¹⁹ and *RAG-2*/*RAG-2*²⁰ genotypes did not display OVA-specific anaphylaxis in this system (0/5 deaths and 0/2 deaths, respectively). Taken together, these observations suggest that non-IgE immunoglobulins may be capable of mediating antigen-specific systemic anaphylactic reactions.

Increased vascular permeability, induced by vasoactive mast cell mediators, is a hallmark of systemic anaphylaxis. To test for the presence of vascular leak, we administered intravenous Evans blue dye to some sensitized mice at the time of OVA challenge²¹⁻²³. Plasma extravasation was assessed by examining the foot pads 20 min after antigen exposure. A marked bluing of the feet of both sensitized wild-type and IgE-deficient animals was observed consistent with a generalized increase in vascular permeability (Fig. 4a). A major vasoactive mediator released by activated mast cells is histamine, and systemic anaphylaxis in humans and rodents has been associated with notable rises in plasma histamine levels^{24,25}. Blood obtained both from wild-type and from IgE-deficient animals 2 min after antigen challenge had marked increases in histamine concentrations (Fig. 4b). This result suggested that mast cell and basophil degranulation is occurring in non-IgE mediated anaphylaxis. This hypothesis was confirmed by direct histological analysis. More than 90% of the mast cells in the ears of both wild-type and IgE-deficient OVA-challenged animals had undergone degranulation (Fig. 4c).

IgE has generally been regarded as the major trigger of immediate hypersensitivity reactions. Recently it has been demonstrated that anaphylactic sensitivity passively transferred with IgE requires that the recipient animals express normal high-affinity

IgE receptors (FcεRI)^{23,26}. Although it is clear that IgE is central to many allergic reactions, some observations have hinted at the existence of alternative pathways of immediate hypersensitivity. Studies of skin hypersensitivity in the mouse using the model of passive cutaneous anaphylaxis have shown that a serum fraction containing IgG can sensitize recipient tissue, albeit in a less potent and relatively shorter-lived fashion than IgE^{17,18}. The findings that mast cells express Fcγ receptors and can be activated by IgG in culture suggest a possible mechanism for the non-IgE response²⁻⁵. In any case, the expression of full-blown systemic anaphylaxis in actively immunized IgE-deficient mice in this study demonstrates clearly that immediate hypersensitivity can be initiated independently of IgE.

Note added in proof: We have recently challenged mice immunized with bovine gamma globulin. Anaphylaxis and death occurred in both wild-type (3/4) and IgE-deficient (4/4) animals, indicating that the phenomenon of IgE-independent anaphylaxis can be observed with protein antigens other than OVA. □

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Reverse occlusion leads to a precise restoration of orientation preference maps in visual cortex

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In the visual system of young kittens, the layout of the cortical maps for ocular dominance and orientation preference converges to an equilibrium state within the first few weeks of life and normally remains largely unchanged. If during the critical period, however, patterned visual experience is restricted to only one eye for a few days, cortical neurons lose their ability to respond to stimulation of the deprived eye. We used the 'reverse occlusion' protocol together with chronic optical imaging to investigate how the profound anatomical changes accompanying monocular deprivation¹ affect the spatial pattern of the cortical orientation preference map. We report here that after 1 week of monocular deprivation, cortical orientation maps for the deprived eye had vanished. But we also discovered that after subsequent reverse occlusion the restored orientation maps were very similar to the original maps. This demonstrates that in spite of functional disconnection of one eye after monocular deprivation, the layout of cortical orientation maps, when re-established for this eye, is not formed from scratch but is strongly influenced by previous experience.

In the developing mammalian visual cortex, monocular deprivation causes the relative influence of the two eyes on cortical

cells to change such that most of the neurons are entirely dominated by the open eye². Anatomically, complexity and length of geniculate-cortical fibres decrease substantially, even if monocular deprivation is maintained for only a few days¹. 'Reverse occlusion' can be used to reverse the effect of the imbalanced visual stimulation: opening the closed eye and closing the initially open eye, during the critical period, causes a complete switch in ocular dominance. After a few days most cells are dominated by the initially deprived eye³⁻⁵. To examine whether the massive reorganization of geniculate-cortical connections occurring in response to short-term reverse monocular deprivation affects the pattern of iso-orientation domains in the visual cortex we used *in vivo* optical imaging of intrinsic signals⁶⁻⁹. Four animals had normal visual experience before the experiments began. At the peak of the critical period (postnatal days (PND) 33 to 40) orientation maps were recorded from a ~4 × 3 mm² region in area 18. Figure 1 shows data from one of these experiments. The dark patches indicate regions strongly activated by the respective stimuli. Iso-orientation domains are patch-like structures with an inter-patch distance of ~1 mm, a pattern very similar to the situation in adult cat visual cortex².

After imaging the orientation maps for each eye the animals were monocularly deprived for 5–9 days by lid suture. Consistent with previous electrophysiological studies^{10,11}, a period of deprivation of more than 6 days caused the activity maps for the deprived eye to disappear (Fig. 1d, e). In one animal in which deprivation was initiated late (PND 40) a faint trace of the original map still persisted for some orientations (data not shown). The eyes were then reverse sutured for 7–11 days. After this period, responses to stimulation of the newly opened eye had reappeared (Fig. 1g, h), whereas responses through the other eye had vanished (Fig. 1p, q). The orientation map for the now open eye was very similar to that observed before deprivation.

To gain a more complete view of the layout of the orientation preference map before, and after reverse occlusion, we calculated 'angle', 'polar' and 'hue-lightness-saturation' (HLS) maps for the initially deprived eye (see legend of Fig. 2 for more details). Before any visual deprivation (Fig. 2a–c), regions of iso-orienta-

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FIG. 1 Activity maps for the right and left eye in a reverse occlusion experiment. *a–i*, Example of orientation maps for the initially occluded (right) eye. *j–r*, Data from the same experiment, but obtained by stimulating the left eye. *a, b*, Activity maps in area 18 for 0° and 90° gratings in a 33-day-old kitten when stimulated through the right eye. After closing the right eye for 6 days, the activity maps have disappeared (*d, e*). When the right eye is opened again (and the left eye closed) activity maps virtually identical to the ones recorded at postnatal day (PND) 33 reappear (*g, h*). *j–r*, Control data obtained for the left eye. During closure of the right eye (between PND 33 and PND 39) the activity maps for the left eye do not change substantially (compare *j* with *m* and *k* with *n*). When the left eye is closed (after PND 39), as expected, the activity maps vanish (*p, q*). For comparison of the maps arrow-heads are placed in corresponding positions in the three respective panels (*a, d, g* and *b, e, h; j, m, p* and *k, n, q*). The right panels show the blood vessel pattern of respective recording sessions. Scale bars, 1 mm.

METHODS. Four kittens were raised normally up to an age of 33–40 days. At this time the animals were anaesthetized, intubated and prepared for an optical imaging experiment using standard procedures¹⁴. The skull of the animal was opened above area 18 at Horsley–Clarke A2–A3. A recording chamber was cemented onto the skull, the dura removed and the chamber was filled with silicone oil. Moving high contrast (black = 0.2 cd m⁻²; white = 8 cd m⁻²) square-wave gratings of four different orientations (0°, 45°, 90°, 135°) with a spatial frequency of 0.15 cycles per degree and a drift velocity of 15° s⁻¹ were presented monocularly on a 19 inch computer screen. A slow scan CCD camera (System 2000, Digital Pixel, UK) was used for imaging intrinsic signals as previously described^{6–9,14}. After the imaging session the right eye was closed by lid suture and the animal was allowed to recover from the anaesthesia. After 5–9 days of monocular deprivation the same animal was anaesthetized and taken into the second recording session for which the closed eye was opened. After the orientation preference maps produced by stimulation of the two eyes were recorded separately, the eyes were reverse-sutured (the right eye was opened and the left eye closed). After a further 7–11 days the iso-orientation maps for both eyes were re-examined in a final experiment.

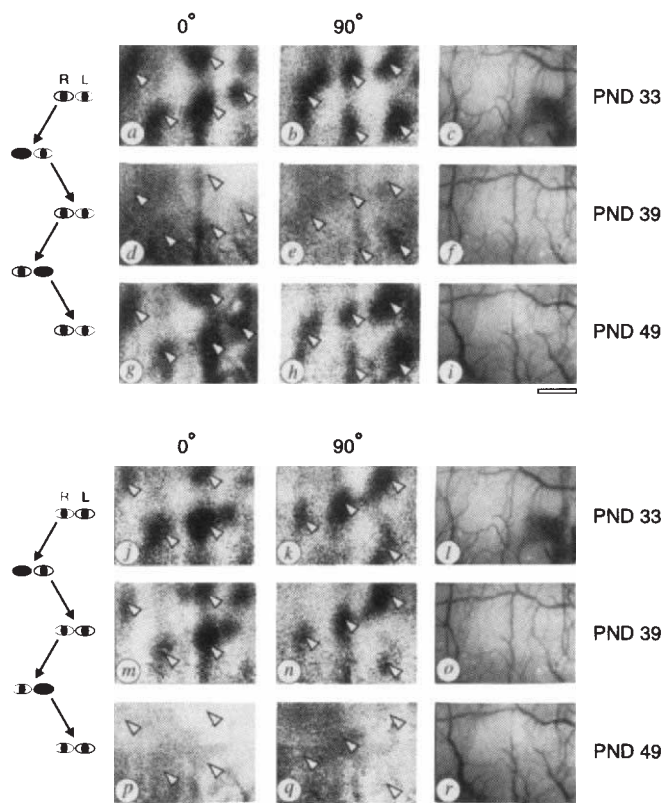
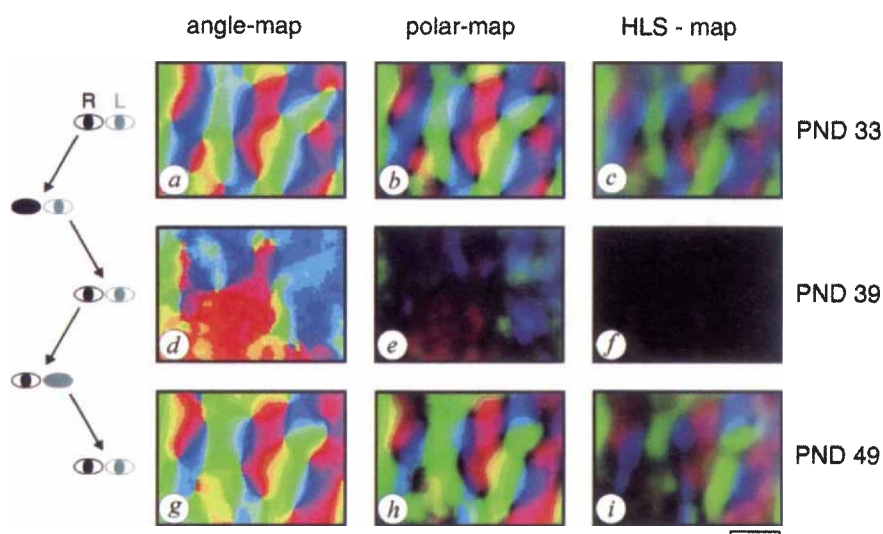


FIG. 2 Angle-maps, polar-maps and HLS-maps for the right eye in a reverse occlusion experiment. For comprehensive analysis of the organization of iso-orientation domains before and after reverse occlusion, angle, polar and HLS maps were calculated. *a, d, g*, Colour code (shown at the bottom) is used to display the preferred orientations for the cortical area shown in Fig. 1. Careful comparison of *a* and *g* shows that after reverse occlusion the original orientation preference map for the deprived eye reappears to a large degree. Because the angle maps are lacking information about tuning and response strength, polar-maps (*b, e, h*) and HLS-maps (*c, f, i*) are shown. *f* in particular demonstrates that indeed activity from the deprived eye is minimal after 6 days of monocular deprivation. Scale bar, 1 mm.

METHODS. Responses to all stimulus orientations were summed vectorially, taking the magnitude of the responses as vector length and the stimulus orientation as their vector angle¹⁴. For angle maps the angle of the resulting vector was then displayed using a discrete pseudo-colour code (0°, yellow; 45°, red; 90°, blue; 135°, green). In polar maps the magnitude of the resulting vector is additionally coded as the lightness of the colour. Because a low magnitude in a polar map can result either from weak overall responses, or from weak orientation tuning (with strong responses for all orientations) we additionally calculated hue-lightness-saturation (HLS) maps in which the hue of the colour codes for the preferred angle, the saturation for the orientation tuning and the lightness for the response strength. In the images of this figure, high frequency noise was reduced using a 3 × 3 Lee-Filter. Note that for Fig. 1 and in the quantitative comparison of Fig. 3, unfiltered data were used.



maps in which the hue of the colour codes for the preferred angle, the saturation for the orientation tuning and the lightness for the response strength. In the images of this figure, high frequency noise was reduced using a 3 × 3 Lee-Filter. Note that for Fig. 1 and in the quantitative comparison of Fig. 3, unfiltered data were used.

tion preference may be thought of as forming 'pinwheels' around orientation centres (Fig. 2*a*), similar to those in older animals^{9,12,14}. Most regions, except for the pinwheel-centres and their immediate vicinity, are well oriented (Fig. 2*b*) and well tuned (Fig. 2*c*). Figure 2*d* shows that the meticulous pattern of

'orientation-centres' and the surrounding pinwheels disappear after the period of initial deprivation. In fact, Fig. 2*e* and *f*, in which the lightness of the colours indicates the response strength, shows that the responses to stimulation of the deprived eye have decreased substantially.

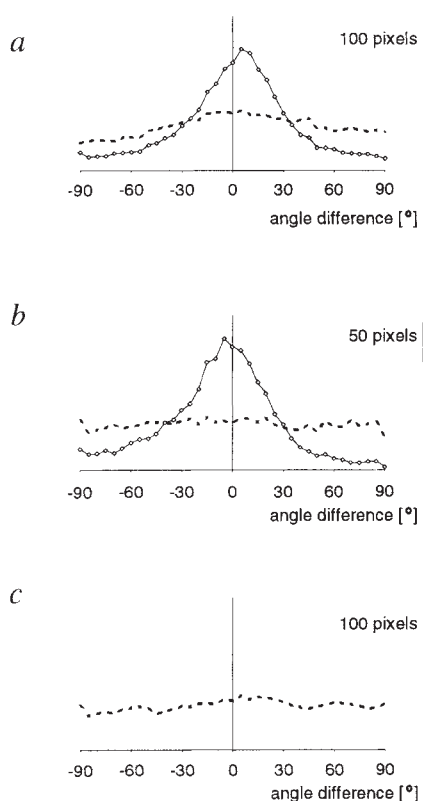


FIG. 3 Quantitative comparison of angle-maps during a reverse occlusion procedure. The histograms show the differences in preferred angles for corresponding points in the orientation preference maps during the various steps of a reverse occlusion procedure. The solid line in *a* shows the quantitative comparison between the maps in Fig. 2*a* and *g*. The dotted line in *a* is a control in which the maps in Fig. 2*a* and *d* are compared. *b*, Corresponding data for another experiment (orientation preference maps not shown). As an additional control, *c* shows the difference between the map in Fig. 2*a* and a recording in a different animal of the same age (orientation preference map not shown). It is evident that the angle difference for the maps obtained in the same kitten before and after reverse occlusion are mostly small (*a* and *b*, solid line), whereas in the various control situations the histograms of angle differences are essentially flat (*a* and *b*, dashed line; *c*). For the calculation of angle maps, data of very high quality are required¹⁴. The data from our other two experiments were not of sufficient quality to yield reliable angle maps. Consequently they could not be used for the quantitative analysis presented in this figure. The single condition maps, however, when analysed in the way shown in Fig. 1, clearly demonstrated that orientation maps similar to the ones before monocular deprivation had re-emerged.

After reversing the occlusion by opening the initially closed eye and closing the open eye, maps very similar to the initial situation re-emerge in response to stimulation of the newly opened eye (compare Fig. 2*a* and *g*, *b* and *h*, *c* and *i*). This is further quantified in Fig. 3 which shows histograms of the differences in preferred orientations between the maps obtained at the various stages of the reverse occlusion protocol (Fig. 3*a*, *b*). For most recording sites the difference in optimal angles between maps before and after the entire procedure is small (Fig. 3*a*, *b*; solid line). In controls, in which maps before the procedure are compared to those after the initial deprivation (Fig. 3*a*, *b*; dashed line) or to a map of another kitten of similar age (Fig. 3*c*), the distributions of angle differences are essentially flat. These data then corroborate the qualitative impression gained from Figs 1 and 2 that, in spite of an intermediate functional disconnection, orientation preference maps are re-established to a remarkable degree after reverse occlusion.

Until recently, the effects of short-term monocular deprivation and reverse occlusion had been thought to reflect mainly synaptic downregulation of inputs from the deprived eye^{10,11,15}. However, 1 week of monocular deprivation is now known to be sufficient to cause substantial anatomical changes in the axonal arbores of geniculo-cortical afferents¹ in area 17. Because it is commonly thought that areas 17 and 18 are organized in a very similar fashion and because orientation preference is thought to be determined by the geometrical arrangement of thalamic afferents^{16,17} it was surprising to find that, despite the massive anatomical changes¹, the layout of orientation preference maps for the initially deprived eye was restored with great precision. This indicates that if cortical maps are initially formed under binocular vision, then disturbed (by monocular deprivation) and allowed to re-establish (by reversed monocular deprivation) within the critical period, the re-formation of these maps does not start from scratch but is strongly influenced by the layout of the initial maps.

The most parsimonious explanation for this observation would be that the vanishing and re-establishment of orientation maps simply reflects synaptic weakening and strengthening of the afferent input remaining present throughout monocular deprivation^{4,5,10,11}. The results of ref. 1, however, argue against this explanation. One remaining possibility, at least for the one animal in which a faint map persisted, is that a sufficient number of the original synaptic connections is retained at least in layer IV^{15,18}, favouring the establishment of new connections from the deprived eye very similar to the original pattern. Alternatively, it is conceivable, though contrary to the view that orientation preference maps are determined by the geometry of geniculo-cortical afferents^{16,17}, that a scaffold for the orientation preference map is laid down in intrinsic cortical circuitry. It has been shown recently that tracing cortico-cortical connections from a site of known orientation preference yields a connection pattern that is elongated along the axis of preferred orientation¹⁹. After reversed monocular deprivation, these intracortical connections, by their mere geometric arrangement, could serve as a seed for re-establishing the orientation preference map.

We are doing further experiments to distinguish between the different mechanisms proposed above. Nevertheless, the present data already show that chronical optical imaging from the cortex of young kittens allows us to approach a question difficult to address with other techniques: how do cortical maps emerge and re-emerge within individual animals and therefore ultimately, how do activity dependent mechanisms influence the precise layout of cortical maps? □

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Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum

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DURING their transit through the endoplasmic reticulum, newly synthesized light and heavy chains of immunoglobulins associate with two endoplasmic reticulum stress proteins. BiP/GRP78, a member of the HSP70 family, binds these polypeptides, presumably through promiscuously exposed hydrophobic sequences^{1,2}, soon after their translocation into the endoplasmic reticulum^{3,4}. GRP94, another endoplasmic reticulum stress protein^{5,6} homologous to HSP90⁷⁻¹¹, also associates with unassembled immunoglobulin chains¹², but its interaction is biochemically, kinetically and structurally distinct from BiP's. We report here that whereas BiP preferentially binds an early disulphide intermediate of light chain and dissociates within a few minutes, GRP94 exclusively binds fully oxidized molecules and dissociates with a half-time of 50 min. These results indicate that GRP94 is itself a chaperone which acts after BiP.

GRP94 associates with unassembled immunoglobulin¹², unassembled major histocompatibility complex (MHC) class II polypeptides¹³ and with a mutant herpes glycoprotein¹⁴, but in each case additional proteins were found in the complex. Thus, it was important to establish whether GRP94 binds polypeptides directly. Sequential immunoprecipitation was used to determine if ternary complexes containing all three proteins exist, or only separate binary complexes (GRP94-Ig and BiP-Ig chain). When labelled proteins eluted from anti-light (L) chain antibody were reprecipitated with either anti-BiP or anti-GRP94, all three components were re-isolated (Fig. 1), demonstrating the existence of ternary complexes. However, BiP and L were enriched relative to GRP94 in anti-BiP eluates, and GRP94 and L were enriched in anti-GRP94 eluates. This suggests that, at least after *in vitro* manipulation, separate BiP-L and GRP94-L binary complexes also exist.

On the basis of band intensities, methionine/cysteine contents, half-lives of each protein and the labelling time, we estimate that 2-4 GRP94 molecules associate with each λ chain. A similar ratio was measured for the binding of glucocorticoid receptor to cytosolic HSP90¹⁵.

To provide additional evidence that GRP94 can bind directly to immunoglobulin chains rather than its association being bridged by BiP, we sought conditions that preferentially stabilize GRP94-Ig chain interactions. Labelled cell lysates were incubated under various conditions for 60 min and only then crosslinked and immunoprecipitated. Three manipulations differentially affected the amounts of GRP94 and BiP coprecipitated with immunoglobulin chain (Fig. 2a). First, inclusion of CaCl_2 in the lysis buffer preferentially enhanced the amount of associated BiP. Second, increasing KCl concentrations greatly destabilized BiP-Ig chain interactions, whereas the association of GRP94 was unaffected. Third, inclusion of exogenous ATP reduced the recovery of BiP whereas that of GRP94 was unchanged (Fig. 2b). Conversely, depletion of cellular ATP before lysis resulted in mostly BiP-heavy (H) chain complexes devoid of GRP94. The reciprocal dependence on ATP level is

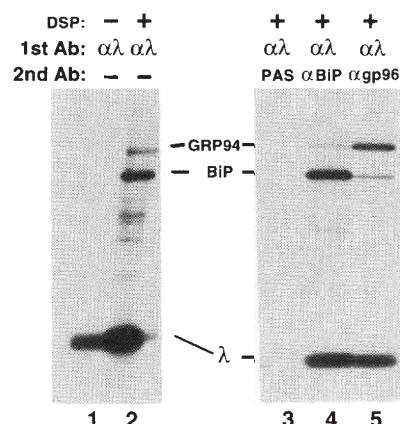


FIG. 1 Sequential immunoprecipitation demonstrating L-BiP-GRP94 ternary complexes and L-GRP94 binary complexes. Non-crosslinked (lane 1) and crosslinked (lane 2) lysates from metabolically labelled cells were immunoprecipitated with anti-L chain-Sepharose. Eluates from the crosslinked lysates were split and reprecipitated with protein A-Sepharose plus no antibody (lane 3), anti-BiP (lane 4), or anti-GRP94 (lane 5). Similar data show ternary complexes of BiP and GRP94 with unassembled H chains.

METHODS. The H chain-negative myeloma MOPC315.37 (clone 37) produces a non-secreted λ I chain²¹. Cells were labelled with ³⁵S-methionine/cysteine (Amersham), crosslinked with DSP (Pierce) and immunoprecipitated as described¹². Rat monoclonal anti-mouse C λ (2B6.1.3; ref. 25) was conjugated to Sepharose 4B (Sigma). Lane 1, to show that this anti-L-Sepharose has no anti-BiP or anti-GRP94 reactivity, cells were lysed in the presence of 5 mM ATP and 50 mM KCl to disrupt L-BiP complexes, and immunoprecipitated with the anti-L-Sepharose. Lane 2, lysates were crosslinked under normal conditions (50 mM bicine, pH 8.0, 40 mM NaCl, 5 mM CaCl_2 , 5 mM KCl, 10 mM Na_2MoO_4 , 1% NP40, and 100 $\mu\text{g ml}^{-1}$ DSP), immunoprecipitated with anti-L-Sepharose, and eluted with 0.2 M glycine, pH 2.5. Equal amounts of this eluate were reprecipitated with protein A-Sepharose (Sigma) plus no antibody (lane 3), rat monoclonal anti-mouse BiP (from D. Bole; lane 4), or rabbit anti-mouse gp96/GRP94 (from P. Srivastava; lane 5). Eluates were analysed by 10% reducing SDS-PAGE.

consistent with BiP's known ATPase activity¹⁶ and with the ability of GRP94 to bind ATP but to hydrolyse it poorly^{17,18,11}. In conclusion, these *in vitro* manipulations demonstrate that GRP94 association can persist under conditions where only a minimal amount of BiP is present. Together with the sequential immunoprecipitation, these data strongly suggest that GRP94 is itself a polypeptide-binding protein.

BiP associates more avidly with non-secreted immunoglobulin chains than with their secreted counterparts^{4,10,12}. Comparison of three myeloma lines shows that the same is true for GRP94 (Fig. 3; ref. 12). Because the association of GRP94 with secreted L chains is limited, we sought additional evidence that it is specific. When expression of stress proteins was induced by 2-mercaptoethanol (2ME)^{5,19}, association of GRP94 (as well as BiP and GRP170²⁰) with wild-type L chains was clearly demonstrated (Fig. 3a). The L chains in these complexes were properly folded despite the earlier reductive stress: they migrated as fully oxidized species, formed some covalent dimers, and were secreted with the same time course as L chains from non-stressed cells (data not shown). We conclude, therefore, that GRP94 can recognize not only mutated but also wild-type L chains.

The λ II chains in clones 26 and 37 differ only by one substitution in the variable domain (Gly 15→Arg), rendering the L chain of clone 37 non-secreted²¹. We sought to determine if GRP94's marked preference for the non-secreted chain is due to the mutant's extended residence in the endoplasmic reticulum (ER) or to its structural alteration. Therefore, the wild-type L chain of MOPC315.26 was arrested in the ER by treating the cells

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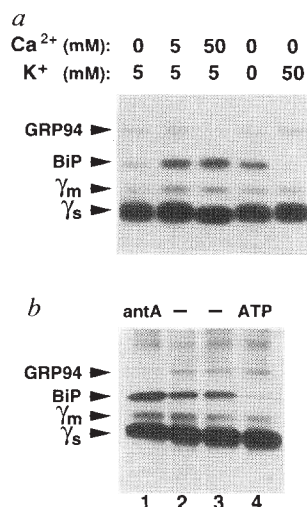


FIG. 2 GRP94 and BiP binding are differentially modulated *in vitro*. **a**, Effect of Ca²⁺ and K⁺. γ -Producing, L chain-negative NSII cells²⁶ were metabolically labelled, lysed in buffer containing 0, 5, or 50 mM CaCl₂ or KCl, as indicated, held on ice for 60 min and then crosslinked and immunoprecipitated with protein A-Sepharose. **b**, Effect of ATP level. Lane 1, NSII cells were incubated with 100 μ M antimycin A (Sigma) for the last 10 min of the 4.5-h labelling period. Cells were lysed in the absence of exogenous ATP (lanes 1–3) or in the presence of 10 mM ATP (lane 4). Lanes 2 and 3 are duplicates of untreated lysates. Lysates were processed as in **a**. γ_s , Secretory γ ; γ_m , membrane γ . Lighter exposures confirmed equal loading of γ -chains. Similar results with respect to ions and ATP were obtained for GRP94 and BiP binding to L chains.

with brefeldin A. Brefeldin A treatment blocked secretion and increased the amount of BiP coprecipitated with secretable L chain, but did not detectably increase GRP94–L association (Fig. 3b). We conclude that retention in the ER and increased BiP binding are not sufficient for binding of GRP94. Rather, GRP94 seems to be sensitive to the structure of the L chain and binds more avidly to the mutant.

The ability to detect GRP94 binding to secretable L chains allowed us to determine how it is related to BiP binding. Two forms of λ can be resolved by non-reducing gels (Fig. 4a); the lower band represents fully oxidized molecules whereas the upper band is a reduced form which is a short-lived ($t_{1/2} < 10$ min) intermediate. BiP associates with both forms, showing a preference for the intermediate as the oxidized form predominates after long labelling. In contrast, GRP94 is associated exclusively with the more mature, oxidized molecules (Fig. 4a). The reduced intermediate is trapped in the presence of 2ME, and is bound almost exclusively by BiP and not GRP94 (Fig. 4b). On further chase without 2ME, the intermediate is progressively converted to the oxidized form; concomitantly, BiP association declines while GRP94 association increases (Fig. 4b). That the two proteins associate sequentially was also demonstrated by short pulse-chase experiments. Both proteins were coprecipitated with λ after only a 1 min pulse, but whereas BiP association was maximal initially and decreased upon chase, GRP94 association increased with time (Fig. 4c). Longer pulse-chase experiments showed that the half-life of GRP94 association was 50 min, whereas the half-life of the BiP–L complex was only a few min (Fig. 4d and ref. 4). Thus, GRP94 associates with secretable immunoglobulin chains after BiP, in transient fashion, but its interaction persists much longer than that of BiP.

These experiments provide functional data about GRP94's role in immunoglobulin biosynthesis and indicate that it acts as

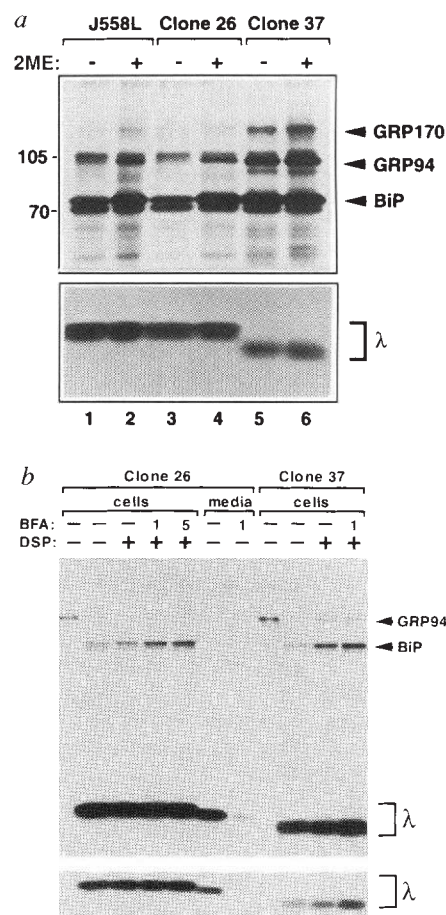
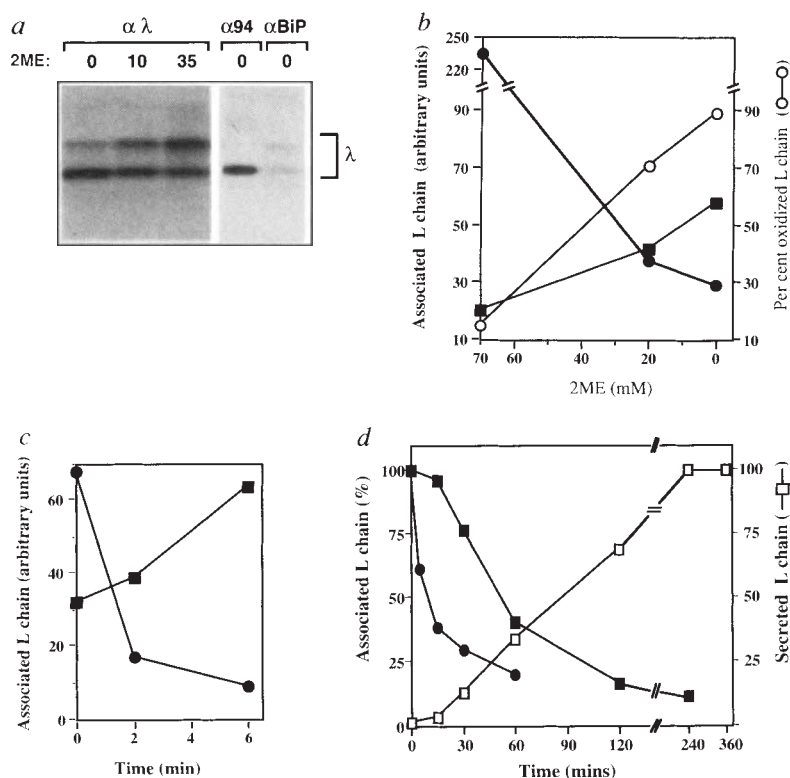


FIG. 3 **a**, GRP94 association with secretable L chains is increased after induction of stress proteins. Three H chain-negative myelomas, J558L (secreting λ l, ref. 4), MOPC315.26 (clone 26, secreting λ II) and MOP315.37 (clone 37), were treated for 4.5 h with medium lacking (–) or containing (+) 35 mM 2ME (Kodak), to induce expression of stress proteins^{5,19}. They were then washed and labelled with ³⁵S-methionine for 4.5 h. Detergent lysates were crosslinked and immunoprecipitated with rabbit anti-mouse λ antiserum (Bethyl) plus protein A-Sepharose. The bottom panel is a 17-fold shorter exposure than the upper panel. The stress protein GRP170 was identified by blotting with an antiserum obtained from J. Subjeck²⁰. The identities of the bands just below BiP (70K) and above GRP94 (105K) are unknown; neither band is serologically related to GRP94 or BiP (data not shown). Note that their amounts were not affected by the 2ME stress, in contrast to BiP and GRP94. Induction of stress proteins by other modes gave qualitatively similar results. **b**, Brefeldin A treatment does not increase the association of wild-type L chain with GRP94. Clone 26 and clone 37 were labelled for 3 h in the presence of 0, 1 or 5 μ g ml^{–1} brefeldin A (Sigma), as indicated. Cells were separated from the media and lysed in the absence (–) or presence (+) of DSP. Cell lysates and media were immunoprecipitated with anti- λ antiserum. In lanes 1 and 8, non-crosslinked lysates were immunoprecipitated with rat monoclonal anti-GRP94 (clone 9G10, StressGen, Victoria, BC, Canada) plus protein G-Sepharose (Pharmacia). The lower panel is a shorter exposure of the λ bands. Brefeldin A was the drug of choice, as it blocks secretion without much effect on intracellular ATP levels²⁷. Note that brefeldin A treatment did not block binding of GRP94 to the non-secretable L chain of clone 37.

a chaperone after BiP. Our data suggest that GRP94 interacts directly with L and H chains. Like BiP, GRP94 binds quite stably to mutated immunoglobulin molecules but transiently to wild-type, secretable chains. However, the conditions that favour association are different for the two stress proteins. Indeed, BiP associates with fewer mature molecules than does GRP94, and it dissociates long before GRP94. GRP94's cytoplasmic homo-

FIG. 4 GRP94 association with L chains is distinct from BiP. **a**, BiP and GRP94 associate with different forms of L chain. MOPC315.26 cells labelled for 15 min in the presence of 0, 10 or 35 mM 2ME, as indicated, were lysed without crosslinking and immunoprecipitated with anti- λ antiserum. In a parallel experiment, MOPC315.26 cells were labelled for 100 min in the absence of 2ME, and non-crosslinked lysates were immunoprecipitated either with anti-GRP94 (9G10) or with anti-BiP, as indicated. Eluates were analysed by non-reducing 12% SDS-PAGE. **b**, Oxidation of reduced L chains leads to GRP94 association and BiP dissociation. MOPC315.26 cells were labelled for 10 min in the presence of 70 mM 2 ME. After removal of a sample, the remaining cells were chased for 10 min in the presence of 20 or 0 mM 2ME. The samples were rapidly chilled in medium containing their respective amounts of 2ME, and the pelleted cells were alkylated with 20 mM *N*-ethylmaleimide. Non-crosslinked lysates were immunoprecipitated with anti- λ and the percentage of oxidized L chains ($\circ$) determined by non-reducing SDS-PAGE. Parallel crosslinked lysates were immunoprecipitated with anti-GRP94 (9G10; $\blacksquare$) or anti-BiP ($\bullet$), analysed by reducing gels, and the amount of co-precipitated L chain was quantified on a phosphorimager. **c**, GRP94 associates with L chain after BiP. MOPC315.26 cells were pulse-labelled for 1 min and chased for the indicated times. Crosslinked lysates were immunoprecipitated with anti-GRP94 (9G10; $\blacksquare$) or anti-BiP ($\bullet$) monoclonal antibodies, analysed by reducing gels, and the amount of co-precipitated L chain was quantified on a phosphorimager. **d**, GRP94 dissociation from secretable L chains occurs subsequently to BiP dissociation. MOPC315.26 cells were pulse-labelled for 10 min and chased for the indicated times. Cells were separated from the medium, crosslinked, and immunoprecipitated with anti-GRP94 (9G10; $\blacksquare$) or anti-BiP ($\bullet$) monoclonals. The amount of coprecipitated L chain displayed on reducing gels was quantified by scanning densitometry of the autoradiogram and normal-



logue, HSP90, chaperones protein folding *in vitro*²² and interacts with immature steroid receptors and with HSP70^{15,23} in a manner analogous to the model proposed here. We propose that in the ER, BiP and GRP94 act in tandem on overlapping populations of folding intermediates, as demonstrated previously for prokaryotic chaperones²⁴, and that GRP94 assists a more advanced step in folding, perhaps aiding the assembly of immunoglobulin subunits. □

Relationship between *Drosophila* gap gene *tailless* and a vertebrate nuclear receptor Tlx

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WE report here the identification of a unique vertebrate nuclear receptor, Tlx, which is expressed exclusively in the neuroepithelium of the embryonic brain. Sequence comparison reveals striking similarity to the product of the *Drosophila* terminal/gap gene *tailless* (*tl*)¹, which is expressed in the embryonic brain and is required for brain development in flies. *In vitro* DNA-binding assays demonstrated that Tlx and Tll proteins share a target gene specificity that is unique among the nuclear receptor superfamily. Ectopic expression of Tlx in fly embryos caused a repression of segmentation comparable to that elicited by Tll. The similarities in structure, expression pattern, target gene specificity and phenotypes in transgenic flies suggest conservation of genetic programs upstream and downstream of this Tlx/Tll class of nuclear receptors during embryogenesis.

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a

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1 AGCGCGCCGCCACCCCGCCACCTCCCGGGGTCGCGAGGCGCTCGGTCGCGGGGAGAGCTCTCTTCGGCTCATTTGGTTTCTTTAGT 90
91 ATTATTTTTCGCTTTCAGCCCTCGCGTTTCGCTTCGGTTTCTTTTTCCTTTTTCCTTTTTCCTTCGCGCTCGCGGG 180
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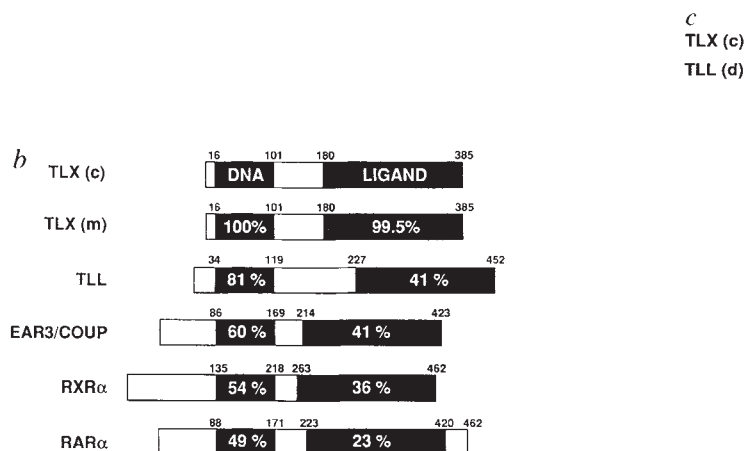
1
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(mouse) 31 41

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451 CGGAGCATTAGGAGGAACAGGACCTAGCTTCGAGTGGGAATCAGGGGGGCTGTCGCGTGGACAGAGCGACAGGAACAGTGGCGG 540
R S I R R N R T Y V C K S G N Q G G C P V D K T H R N Q C R
81 91 101
541 GCCTGCCGGGTGAAGAAGTGTGGAAGTCAACATGAACAAGACGTGTGACGACAGAGCGGGGCCACAGTACATCAACAATACGGAAAG 630
A C R L K K K C L E V N M N K M D A V Q H E R G P R T S T I R K
111
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171 181 191
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201
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L L F M S I K W A K S V P A F S T L S L Q D Q L M L L E D A
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N T D S Q K L N L I I S E I Q A L Q E V V A R F R Q L R L D
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2611 ACTGAGAAAAATCAGCACTTGGACTATCATAGATGATTAACCTTTCTGTACAAAGTGATTAACGATTGTTGAAGTTAAAGATT 2700

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FIG. 1 Nucleotide and predicted amino-acid sequences of clone X31 encoding chick Tlx compared with other nuclear receptors. **a**, Complete nucleotide sequence of a 2.7 kb insert from X31. Conceptual translation begins with the methionine codon at nucleotide 328 and ends with a stop codon at 1,483. The predicted amino-acid sequence of chick Tlx is aligned with that of mouse Tlx which was also deduced from a cDNA sequence. Identical amino acids are indicated by dashed lines and substitutions are boxed. Solid arrows demarcate the DBD and open arrows the LBD. **b**, Comparison of chick Tlx amino-acid sequence with mouse Tlx and other nuclear receptors. 'DNA' denotes DNA-binding domain and 'LIGAND' the ligand-binding domain. The % identity is indicated in the aligned sequences. **c**, Amino-acid sequence alignment of chick Tlx and fly Tll. The DBDs and LBDs are shaded. Functionally important subregions in the DBD are boxed and labelled. Tlx and Tll contain P box sequences that differ from the consensus sequence (EGCKG) such that both encode aspartic acid (D) instead of glutamic acid (E); in addition, a lysine (K) that is absolutely conserved in all other members is substituted with either serine (S) or alanine (A). The D box encodes 7 instead of the usual 5 amino acids. Sequence identity extends beyond the Zn-finger region into the T/A box.

METHODS. A random primed 1.8 kb *EcoR*I–*Pst*I fragment of mRXR β cDNA² was used to screen a λ ZAPII 3-day chick embryonic cDNA library¹⁵. Filters were washed at low stringency ($2\times$ SSC, 50°C). Positive clones were purified, the plasmid DNA recovered, and partial sequences were determined using synthetic primers¹⁸ to classify the clones. The nucleotide sequence of clone X31 was completely analysed in both strands. An internal *Sma*I–*Nhe*I fragment of X31, encoding the DBD of chick Tlx was used to screen a day-11.5 mouse embryonic cDNA library prepared in λ ZAPII¹⁵. Filters were washed at high stringency conditions ($0.5\times$ SSC, 58°C) and 4 overlapping clones were isolated. One full-length clone (M6) was used to determine the nucleotide sequence. Sequence alignment and comparison was done with BESTFIT program in the UW GCG program package¹⁹.



c

TLX (c) TLX (d)

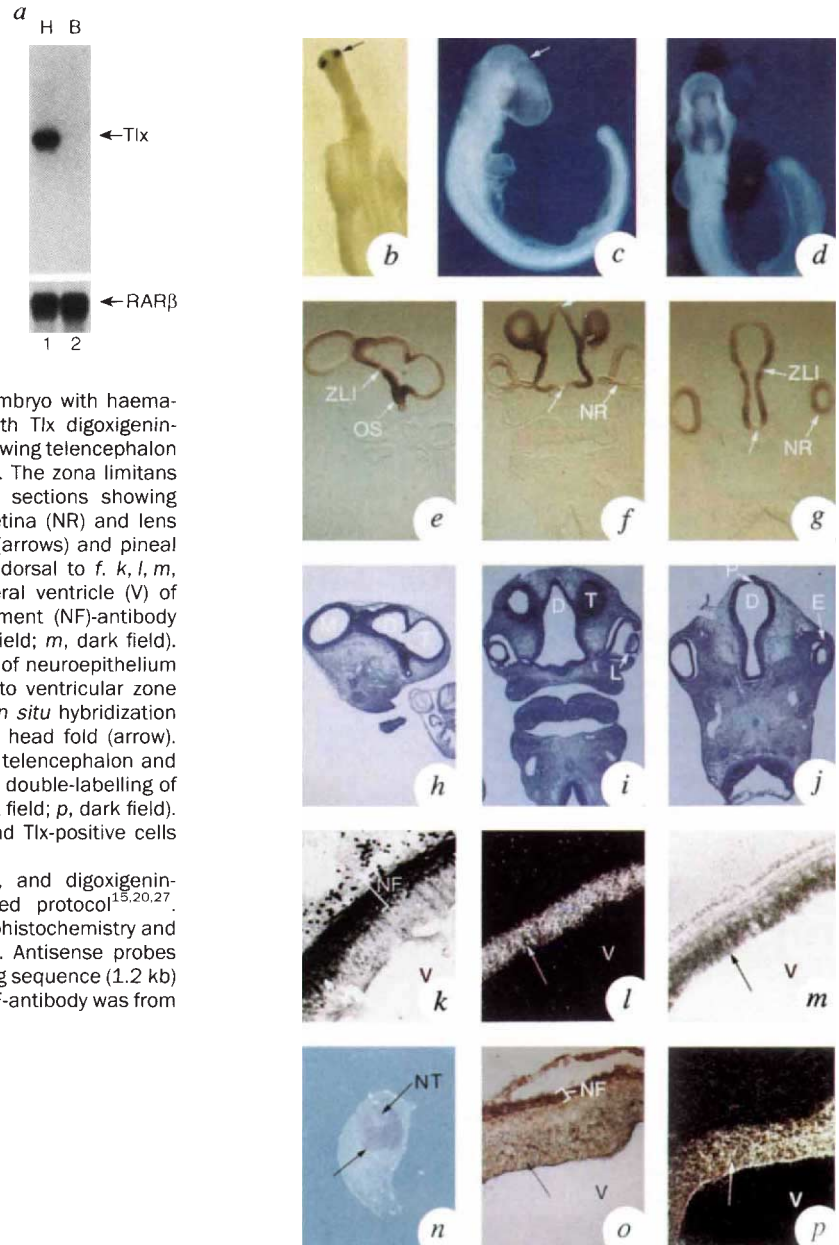
DNA binding domain

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1 MSKPAGSTSRIL...DIPCKVGGDRSSGKHVGYA 32
1 MQSSEGSFDMMDQKYNVRLSPAASSRILYHVPCKVCRDSSGKHVGYA 50
31 DPCCSTFKRSIRRNNTYVSGNQCQVPOVXTHRNOACRLKKCLEVN 82
53 CDGCAGFKRSIRRSROYVCKSQKQGLCVVVKTHRNOACRLKKCFKVG 100
83 MKDAVHERGPRSTSTRE...VALYFRGHKEESSGAPHFATALPAPAFF 132
101 MKDAVHERGPRSTSTRE...MAMY...KDMMGAGMFPQIPAEILMNTAALT 149
133 AVSQL...EPHGLELAAVAGTPERQALVGLAQTPKYPHEVN 171
150 GFPGVPMFPLGPRAGHHPAHMAAFQPPSAAVLDLSVPRPHVPHQ 199
172 G...TPMYLYE...VATESVCSAAARLLEMSIKWAS 202
200 GHHGFTPTAAYMNAALRALPPTPPLMAAHLKETAHLKKNVWIKS 249
203 VFAPSTLSLQDQLLEDAMRELFLVGLIAQWAPVDANTLLAVSGNDGN 252
250 VFAPSTLNPDLQLLEESWKEFFILMAQYLMFMFAQLLEV...YESEN 297
253 TDSQKLNKIISEIQALQEVVARFRQLRLDTEFAFLKCIIVTKRVP... 298
298 ANREINGMVTREVNATQEVNLQDCHLNDSTYECRLAISLFKSPSPAS 347
299 ...TESGS...ELRSFNAAIAALQDEAQLTINSYH 330
348 STEDIANSSILTGSGPNSSASASGSLGSKVAMHNDANSALENIQ 397
331 TRYPTOPCRFGKLLLPALRSISPSSTIEVFFKKTIGNPITLLSDMY 380
398 RTHPSQPMFTQTLGVQMLHKVSSFTIEELFKRTIGDITVRLSDMY 447
381 KSSDI* 385
448 SQRKI* 452

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FIG. 2 Expression of *Tlx* mRNA in chick and mouse embryos. **a**, Northern analysis of mRNA from day-5 chick embryo heads (H, lane 1) and bodies (B, lane 2) using *Tlx* and *RAR β* cDNA as probes. **b**, Dorsal view of a stage 11 chick embryo subjected to whole mount *in situ* hybridization with *Tlx* probe. Signal is restricted to anterior end of the embryo with well defined expression in the primary optic vesicles (arrow). **c**, Lateral view of stage 16 chick embryo showing localization of *Tlx* signals in forebrain and eye. The sharp margin of expression at fore-brain-midbrain border is indicated (arrow). **d**, Frontal view of **c**, showing graded regions of expression in telencephalon, diencephalon (see text). Midline (arrows) is devoid of expression. **e-j**, Adjacent sections from stage 21 chick embryo with haematoxylin-staining (**h**, **i**, **j**) or *in situ* hybridization with *Tlx* digoxigenin-labelled probe (**e**, **f**, **g**). **e**, **h**, Parasagittal sections showing telencephalon (T), diencephalon (D), midbrain (M), optic stalk (OS). The zona limitans intrathalamica (ZLI) is indicated. **f**, **g**, **i**, **j**, Coronal sections showing telencephalon, diencephalon, and eye (E). Neuroretina (NR) and lens (L) are indicated. Signals are absent from midline (arrows) and pineal gland (P), as well as ZLI. Level of section in **g** is dorsal to **f**. **k**, **l**, **m**, Adjacent sections through telencephalon and lateral ventricle (V) of an 8-day-old chick embryo showing anti-neurofilament (NF)-antibody staining (**k**) or 35 S-labelled antisense *Tlx* (**l**, bright field; **m**, dark field). NF-positive cells are localized in outer mantle layer of neuroepithelium (bracket), whereas *Tlx*-positive cells are restricted to ventricular zone (arrow). Ventricle (V) is indicated. **n**, Whole-mount *in situ* hybridization of day-8 mouse embryo showing defined signal in head fold (arrow). Neural tube (NT) is indicated. **o**, **p**, Section through telencephalon and lateral ventricle of day-11.5 mouse embryo showing double-labelling of NF-antibody and 35 S-labelled antisense *Tlx* (**o**, bright field; **p**, dark field). NF-positive cells (bracket) in outer mantle layer and *Tlx*-positive cells (arrow) in ventricular zone are indicated.



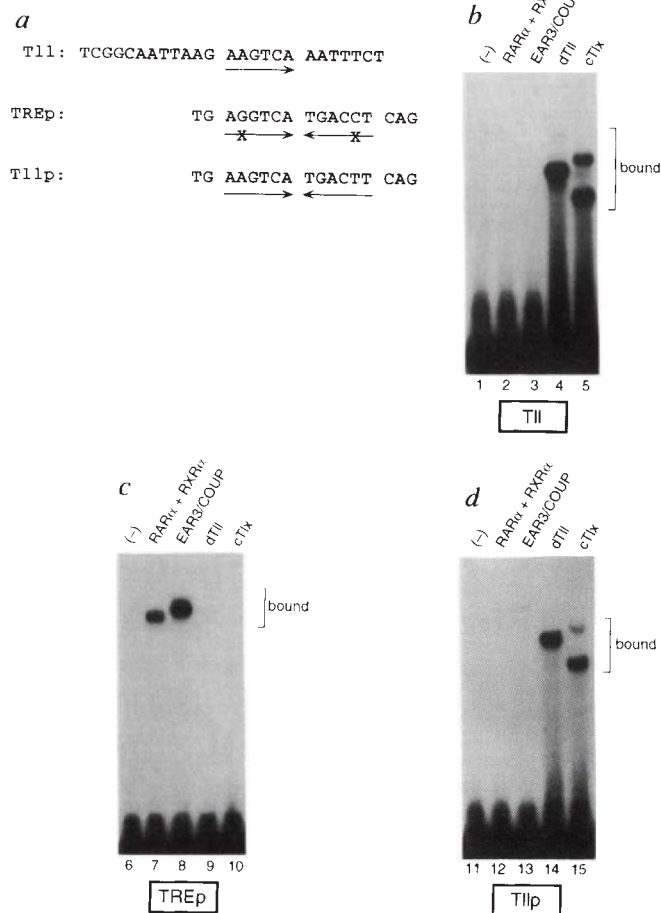
Low-stringency screening of a chick embryonic complementary DNA library with a mouse *RXR β* cDNA² fragment, resulted in positive clones of which most encode chick homologues of mammalian RXRs^{2,3}, COUP-class proteins^{4,5}, and two overlapping clones that encode a novel nuclear receptor, which we termed *Tlx* (Fig. 1a). Sequence comparison with RXRs revealed only marginal identity; the highest degree of similarity was found with the product of *Drosophila* gene *tailless* (*tll*)¹, notably in the DNA-binding domain (DBD) and to lesser extent in the ligand-binding domain (LBD) (Fig. 1b). Changes in two discrete subregions in the DBD, the P box and D box, which modulate the mode of DNA binding^{6,7}, distinguish the fly *Tll* from all other nuclear receptors. The first change is that an otherwise absolutely conserved lysine is replaced by an alanine in the P box, and the second that the D box encodes 7 amino acids instead of the usual 5 (ref. 1). Strikingly, the altered lysine and the expanded D box are also found in *Tlx* (Fig. 1c). In addition, the T/A box which is implicated in dimerization and/or sequence recognition is conserved⁸ (Fig. 1c). In the LBD, *Tlx* shows a comparable degree of similarity to both *Tll* and human *EAR3/COUP-TF*^{4,5} (Fig. 1b). To examine whether these structural features are conserved among vertebrates, we isolated a

mouse *Tlx* cDNA. Deduced amino-acid sequence of the mouse *Tlx* revealed remarkable conservation between avian and mammalian proteins (Fig. 1a, b). Among the 12 amino-acid substitutions, 11 are clustered in the hinge region where the fly *Tll* shows no significant similarity to the chick protein (Fig. 1a, c).

During fly embryogenesis, *tll* seems to play two distinct roles, one in the establishment of non-metameric domains at the embryonic termini, and the second in the development of the nervous system. Zygotic transcripts are initially detected at both embryonic termini, but after cellularization become localized in the developing brain¹. To define the localization of vertebrate *Tlx* transcripts, day-5 chick embryos were grossly divided into heads and bodies for RNA extraction. Northern blot shows a major 3.2 kilobase (kb) *Tlx* transcript present only in the head fraction (Fig. 2a). To examine further the expression, we used *in situ* hybridization and found that *Tlx* expression is first detectable at low levels in the head ectoderm at stage 8⁹ (not shown). Earlier stages (2–7) were examined both by whole-mount *in situ* hybridization and by reverse transcription-polymerase chain reaction, but no expression was detected (not shown). By stage 11, a clearly defined region of expression is found in the primary optic vesicles (Fig. 2b). At stage 16, *Tlx* is expressed

FIG. 3 *In vitro* DNA binding specificity by Tlx protein. **a**, Sequences of oligonucleotides used in DNA-binding assays. 'Tll' encodes a hexameric Tll binding site (indicated by underlying arrow) which was identified in the *Krüppel* gene enhancer region¹³. 'TREp' is a palindromic sequence which is a common binding site for several nuclear receptors²². 'Tllp' is derived from TREp by changing one nucleotide in the consensus hexameric half-site 'AGGTCA' to the *tll* half-site 'AAGTCA'. The critical difference between TREp and Tllp is indicated by an X under the TREp sequence. **b–d**, DNA binding patterns of *in vitro* translated proteins to probes indicated in the boxes. Lanes 1, 6, 11, blank reticulocyte lysate added to the binding reactions (–). Lanes 2, 7, 12 contain a mixture of human RAR α and human RXR α to allow heterodimer formation. Lanes 3, 8, 13 were incubated in presence of human EAR3/COUP-TF. Likewise lanes 4, 9, 14 and lanes 5, 10, 15 contain fly Tll and chick Tlx proteins, respectively. The specific protein–DNA complex is indicated by bracket 'bound'. An identical binding pattern is seen with proteins generated with wheat-germ extract.

METHODS. Double-stranded oligonucleotides encode 4-nucleotide overhangs (5'AGCT3') at 5' ends and were labelled by filling reaction with Klenow enzyme in presence of α -³²P-dCTP. The proteins were synthesized by coupled *in vitro* transcription–translation system (TNT, Promega) using T7 RNA polymerase and rabbit reticulocyte lysate. The amount of input protein was adjusted by monitoring incorporation of ³⁵S-methionine into the full-length protein. To increase efficiency of *in vitro* translation, chick Tlx cDNA was modified to encode an optimized translation start site (ACCACC-ATG)²⁸. Binding reactions were as reported²².



predominantly in the forebrain and eye (Fig. 2c, d). Sections of stage 21 embryos revealed Tlx-positive cells in the neuroretina, optic stalk and dorsal midbrain in addition to the neuroepithelium of the forebrain with strongest expression in the telencephalon and D1/D2 and D4 regions of the proposed diencephalic neuromeres¹⁰ (Fig. 2e–j). The midline cells, pineal gland and region corresponding to the zona limitans intrathalamica are devoid of Tlx expression. Anti-neurofilament antibody was used to distinguish differentiated neurons in the outer mantle layer of the telencephalic neuroepithelium in day-8 embryonic brain sections (Fig. 2k). Tlx-positive cells are restricted to the ventricular (proliferating) zone from which neurofilament-positive cells are absent (Fig. 2l, m). The pattern of Tlx expression in mouse embryos is comparable to that observed in chick. In early embryos, Tlx expression can be detected in the head ectoderm (day 7.5, Fig. 2n), and at later stages, cells expressing Tlx are localized in the ventricular zone of the neuroepithelial layer (Fig. 2o, p). These observations suggest that Tlx is involved in the transcriptional control in undifferentiated neuroepithelial cells in the anterior regions of the developing vertebrate brain. This expression pattern closely parallels the postgastrulation expression of fly *tll* which is predominately observed first in the region corresponding to procephalic neuroblasts and later in the optic lobes¹. Note that in some fly *tll* mutants, the optic lobe and most of the nerve cells that constitute the brain are missing^{1,11}.

Tll binds upstream regions of target genes such as *Krüppel* (*Kr*) and *knirps* (*kni*), in which a common hexameric sequence of AAGTCA is found^{12,13}. Using gel mobility shift DNA binding assays we compared the ability of Tll and chick Tlx to bind specific DNA sequences (Fig. 3). RAR α and RXR α (which form heterodimers)¹⁴ and EAR3/COUP-TF (which forms homodimers)^{4,5} were included for comparison. In the presence

of a Tll binding site in the *Kr* enhancer¹³, specific binding was observed with Tll and Tlx but not with RAR–RXR heterodimers or COUP, demonstrating that Tlx can recognize the natural Tll site (Fig. 3a, b). To increase the potential for RAR–RXR heterodimers and COUP homodimers to bind the Tll sequence, we prepared oligonucleotides encoding artificial palindromes of either AAGTCA (Tllp) or AGGTCA (TREp)²² (Fig. 3a). A mutually exclusive pattern of half-site recognition was seen; RAR–RXR and COUP bound only TREp, and Tlx and Tll only Tllp (Fig. 3c, d). Both Tlx and Tll can generate two complexes with different mobilities, which seem to correspond to monomeric and dimeric forms. Tlx shows a higher tendency to form the slower complex than does Tll, which may be accounted for by the increased similarity to EAR3/COUP-TF in the LBD, which can stably form homodimers (Fig. 1b). An identical pattern for DNA-binding specificity and complex formation was observed with mouse Tlx (not shown). The guanine at position two in the half-site is highly conserved among nuclear receptor response elements. The altered preference of Tlx and Tll identifies a novel target site specificity which is probably due to the substitution of the conserved lysine in the P box recognition helix^{6,7}.

The resemblance in structure, pattern of expression, and target gene specificity suggests that Tlx may be able to regulate genetic cascades during early embryonic development in a manner similar to fly *tll*. To test this hypothesis we created transgenic flies expressing Tlx under the control of an hsp70 promoter. Ectopic expression of *tll* under the hsp70 promoter induces loss of metameric segments accompanied by alteration of target gene expression¹⁶. Induction of HS-Tlx in embryos resulted in denticle fusion and repression of segmentation, accompanied by repression of reported Tll target genes, *kni* and *engrailed* (*en*)

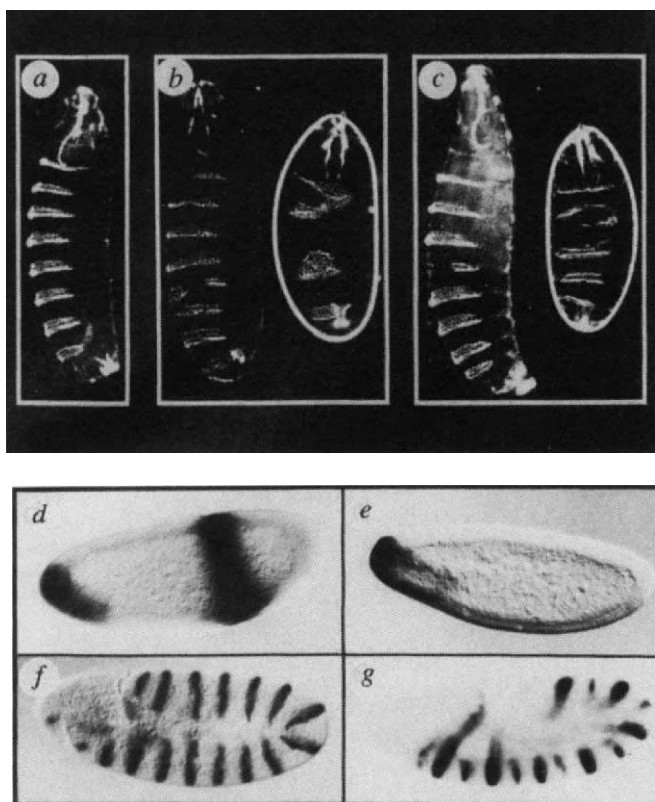


FIG. 4. Ectopic expression of chick Tlx in fly embryos mimics the action of Tll in repression of segmentation and alteration of target gene expression. *a-c*, Cuticle preparations of embryos aged 1.5–2.5 h (stages 3–5)²⁴ at initiation of heatshock (10 min at 37 °C). *a*, Wild-type embryo (Canton-S); *b*, HS-Tlx embryos (from HSTl4); *c*, HS-Tlx embryos (from HSTl6). The most sensitive segments appear to be abdominal segments 3, 4 and 5. *d-g*, Whole mount *in situ* hybridization of HS-Tlx embryos. *d, e*, Embryos were 1.5–2.5 h old at initiation of heatshock (30 min at 37 °C), fixed 30 min later and hybridized with *kni* probe. *f, g*, Embryos were 3.0–3.5 h old when they were heatshocked, and hybridized with *en* probe. *e, g*, Wild-type embryos with *kni* and *en* probes, respectively.

METHODS. Full-length coding regions of chick Tlx and fly *tll* were placed into a pUChsneo vector and P element injection and transformation procedures were as described²⁵. G1 offspring were selected on 250 µg ml⁻¹ G418 and crossed to Canton-S to increase stocks. Three independent lines each of HS-Tlx and HS-*tll* were generated. Cuticles were prepared as previously described²³ and examined under darkfield microscopy. Embryos were processed and antisense digoxigenin probes transcribed for whole mount *in situ* hybridization as described²⁶.

(Fig. 4), demonstrating that Tlx can mimic the action of Tll *in vivo*. This indicates that Tlx contains the information and structures necessary for Tll function, despite the limited similarity in the LBDs. At present, it is not clear whether there is a common ligand for both receptors or if they are ligand independent. The striking parallels between Tlx and *tll* expression and function further implicate the conservation of fundamental signalling pathways between vertebrates and invertebrates during brain development, as has been shown for homeobox genes¹⁷. The strictly localized and conserved patterns of expression may contribute to the establishment of regional identity during embryogenesis through the mediation of extracellular signals to regulatory gene networks through this class of nuclear receptors. □

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Proton migration along the membrane surface and retarded surface to bulk transfer

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SINCE the proposal of the chemiosmotic theory¹ there has been a continuing debate about how protons that have been pumped across membranes reach another membrane protein that utilizes the established pH gradient. Evidence has been gathered in favour of a 'delocalized' theory, in which the pumped protons equilibrate with the aqueous bulk phase before being consumed, and a 'localized' one, in which protons move exclusively along the membrane surface^{2,3}. We report here that after proton release by an integral membrane protein, long-range proton transfer along the membrane surface is faster than proton exchange with the bulk water phase. The rate of lateral proton diffusion can be calculated by considering the buffer capacity of the membrane surface. Our results suggest that protons can efficiently diffuse along the membrane surface between a source and a sink (for example H⁺-ATP synthase) without dissipation losses into the aqueous bulk.

In *Halobacterium salinarum*, light-induced ATP synthesis occurs without a measurable change of protonmotive force under dim light conditions⁴. We used the well characterized purple membrane of *H. salinarum*, which is a two-dimensional

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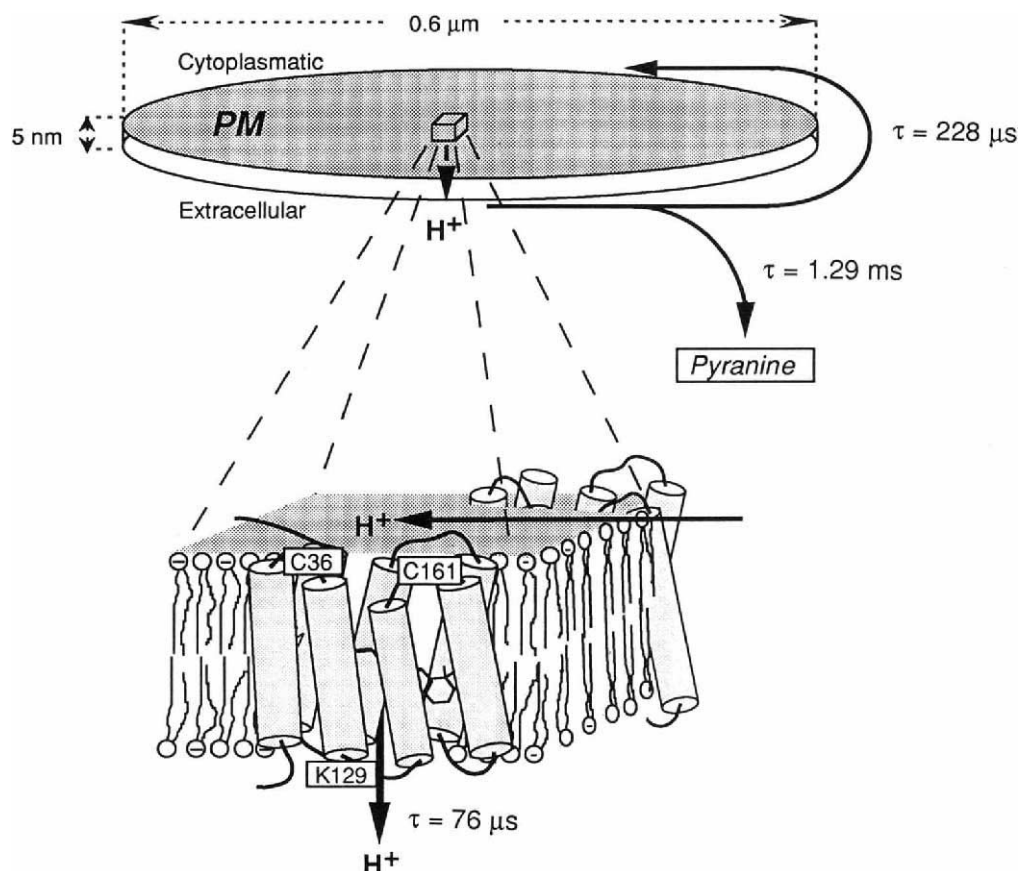


FIG. 1 Purple membrane (PM) with the integral membrane protein bacteriorhodopsin and attachment sites of the pH-indicating dye fluorescein (boxed). Pyranine detects pH changes in the bulk water phase. Time constants τ were: for proton release to the extracellular membrane surface, 76 μs ; for lateral proton transfer, 228 μs ; and for surface to bulk transfer, 1.29 ms.

crystalline array of the integral membrane protein bacteriorhodopsin with lipids. When energized by light, bacteriorhodopsin undergoes a photocycle with several intermediate states. A proton is released to the extracellular surface during formation of the longest-lived intermediate (M)^{5,7}, followed by proton uptake from the cytoplasmic side. We analysed the pathway of the released protons using the planar purple membrane as an open membrane system.

We detected proton transfer both spatially and time resolved (Fig. 1). Fluorescein bound to Lys 129 of bacteriorhodopsin⁶ monitored light-induced proton release at the extracellular surface. At the cytoplasmic surface fluorescein was linked to various cysteine residues. Pyranine, a highly water soluble pH indicator^{5,8}, monitored proton migration in the bulk water phase. Figure 2 shows light-induced temporal and spatial changes in the proton concentration at 20 °C. The trace in Fig. 2a originates from a sample where native bacteriorhodopsin was labelled with fluorescein at the extracellular surface. Fitting a sum of 3 exponentials to the data yields a time constant of $\tau = 76 (\pm 18) \mu\text{s}$ for the appearance (negative change in absorbance) of the released protons at the extracellular surface (upward arrow in Fig. 2a). Pyranine detected the protons with $\tau = 1.29 (\pm 0.47) \text{ ms}$ which reflects the transfer of the released protons from the membrane surface into the aqueous bulk (downward arrow in Fig. 2d). Fluorescein at the cytoplasmic surface monitored the released protons with $\tau = 228 (\pm 39) \mu\text{s}$ (upward arrows in Fig. 2b, c; horizontal arrows indicate subsequent proton uptake by bacteriorhodopsin), with no difference for different locations of fluorescein on the cytoplasmic surface (amino acid 36 in 2b; position 161 in 2c; or position 38, not shown).

The faster protonation of fluorescein at the cytoplasmic surface compared to that of pyranine in the bulk is surprising as the protons must migrate macroscopic distances (the mean diameter of a purple membrane patch is about 600 nm and the mean distance of a bacteriorhodopsin molecule to the rim is 122 nm). Calculating the number of protons detected by each

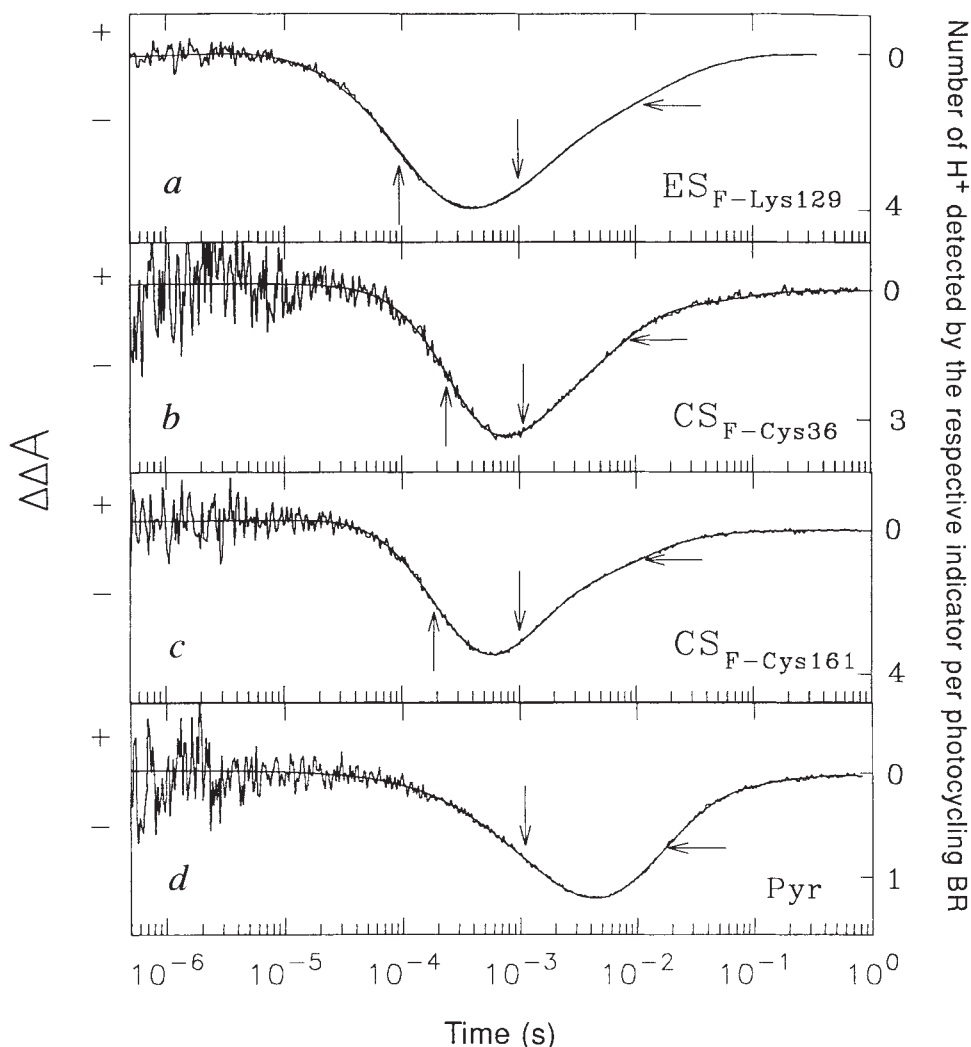
pH indicator per photocycle shows that all the protons released to the extracellular surface are detected by fluorescein bound to the cytoplasmic surface. We conclude that after a light-induced pH jump there is fast and quantitative equilibration between the extracellular surface and the cytoplasmic reaction long before the pH of the bulk water phase is affected. Shunting the released protons across the purple membrane is excluded by the low proton permeability of the membrane^{9,10}. The number of translocated protons per bacteriorhodopsin is about one, as determined by pyranine in the aqueous bulk phase⁵ (Fig. 2d). The higher stoichiometry for the protonation reaction of the surface-bound fluoresceins indicates a smaller reaction volume, as pH equilibration between both membrane surfaces is faster than surface to bulk transfer⁶.

Because of the faster lateral proton migration, surface to bulk transfer of released protons is not only detected by pyranine, but also by fluorescein, no matter which side it is bound (downward arrows in Fig. 2). Mobile buffer molecules can accelerate surface to bulk transfer by collisional proton transfer^{5,6,8,11,12}. The time constants for both surface-bound fluoresceins and pyranine in the aqueous bulk exhibit the same buffer dependence. For example, increasing the concentration of mobile buffer (imidazole) from 0 to 1 mM decreases the time constant of surface to bulk transfer from 1.29 ms to about 300 μs . The same buffer (but not Tris, for example) prevents light inhibition of respiration¹³ in living halobacterium, indicating that in growth medium no mobile buffers prevent surface-localized proton flow (G. Krippl and D.O., unpublished results). These findings and those of ref. 4 show the biological relevance of the observed proton transfer at the membrane surface, at least for this unicellular organism.

From the temperature dependence of the rate constants (Fig. 3), the activation energies for the proton transfer steps were calculated according to the Arrhenius equation. The protonation reaction for fluorescein bound to Cys 36 at the cytoplasmic surface (lateral proton transfer) has an unusually high activation

FIG. 2 Flash-induced transient absorption changes (20 °C, 150 mM KCl, pH 7.5). *a*, Fluorescein (F) covalently bound to Lys 129 at the extracellular surface (ES) of wild-type bacteriorhodopsin (BR); *b*, Fluorescein at Cys 36 at the cytoplasmic surface (CS). *c*, Fluorescein at Cys 161 at the CS. *d*, Pyranine (Pyr) dissolved in the aqueous bulk phase of the fluorescein-labelled D36C mutant. The right ordinate indicates the number of protons detected by the respective pH indicator per photocycling BR. Upward arrows correspond to the time constant for the protonation reaction of surface-bound fluorescein. Downward and horizontal arrows indicate time constants of surface to bulk transfer and subsequent proton uptake by bacteriorhodopsin, respectively. Negative absorption changes correspond to transient pH decrease. Smooth lines represent multiexponential fits to the data traces.

METHODS. Selective labelling of Lys 129 was achieved using fluorescein-5-succinimidylester⁶. Cys-mutants of BR were prepared according to ref. 19. Labelling of the Cys-mutants was by reaction of 5 mM fluorescein-5-maleimide (FMI) or 5-iodoacetamidofluorescein (IAF) with 112 μ M point-mutated BR in the presence of 1 mM EDTA, 150 mM KCl, 336 μ M DTT, at pH 7.0 (FMI in dimethylformamide (DMF)) or pH 8.0 (IAF in buffer or DMF). With stirring under an argon atmosphere, samples were left overnight at room temperature or at 37 °C for 1 h, followed by washing with 150 mM KCl (twice) and dialysis (for a similar approach with heterologous expressed BR monomers in detergents, see ref. 20). Selective labelling, 0.7–1 fluorescein per Cys-mutated BR, was checked by site-specific proteolysis⁶. Transient absorption measurements (20 ns time resolu-



tion) were done at $\lambda = 495$ nm for fluorescein and 465 nm for pyranine. PM was isolated at low ionic strength by centrifugation²¹.

energy of 88 kJ mol⁻¹ (circles in Fig. 3). This is compared to 40 kJ mol⁻¹ for the protonation of fluorescein at Lys 129 (intraproteinous proton transfer, squares in Fig. 3) or to 54 kJ mol⁻¹ for the protonation of pyranine (surface to bulk transfer in the D36C mutant, diamonds in Fig. 3; a similar activation energy was determined for wild-type bacteriorhodopsin⁶). The high activation barrier might originate from the proton transfer from the extracellular surface to the cytoplasmic surface around the edge of the purple membrane fragment. This reaction is rate-limiting, which accounts for our experimental result that the time constant for the protonation of fluorescein bound to the cytoplasmic surface could not be accelerated by reducing the size of the purple membrane patches from about 0.6 μ m to 0.2 μ m by sonification and/or size fractionation. Because of different activation energies and small differences in time constants, the intraproteinous proton transfer and the subsequent proton transfer to the cytoplasmic surface approach the same reaction rate at higher temperatures (Fig. 3).

Our results show that a pH sensor positioned at the membrane surface, at an average of 240 nm from the proton releasing site, detects the liberated protons eight times faster (at 20 °C) than the pH probe in the bulk water phase at an average of only 17 nm from the proton ejecting bacteriorhodopsin (Figs 1 and 2). The long dwell-time at the membrane surface at low and high ionic strength (up to 3.7 M KCl was examined) allows protons to migrate long distances before equilibration with the aqueous bulk occurs. This lateral proton transfer is not a pecu-

liar property of the purple membrane system, but is also observed in reconstituted vesicles composed of different lipids and monomeric or aggregated bacteriorhodopsin¹⁴. Fast lateral proton motion along the surface does not require a larger diffusion coefficient than in bulk water, as has recently been put forward³ for H⁺ diffusion along lipid monolayers (and which is highly disputed^{12,15,16}). Protons with a diffusion coefficient as determined in bulk water (9.3×10^{-5} cm² s⁻¹)¹² would diffuse within 1.5 μ s from the extracellular surface to the cytoplasmic surface assuming a mean distance for the two-dimensional diffusion of 240 nm. This is much faster than the difference (152 μ s) between the experimentally determined appearance of the released protons at the extracellular surface and at the cytoplasmic surface (Fig. 2). At higher temperatures, the rate of proton transfer along the surface approaches that of proton liberation from the bacteriorhodopsin outlet, indicating that lateral H⁺ diffusion comes close to the diffusion rate of protons in pure water. Retarded surface to bulk transfer is still observed at higher temperatures (diamonds in Fig. 3).

The origin of the fast diffusion along the surface and the slower proton dissipation into the bulk phase might be the interplay of protonation/deprotonation reactions of amino acids, as well as lipid head groups (manifested by a high buffer capacity⁹), and proton transfer steps via water molecules restrained at the membrane surface¹⁷. This extended Coulomb cage will lead to large proton polarizability¹⁸. A Monte-Carlo calculation based on an apparent diffusion constant for protons in purple mem-

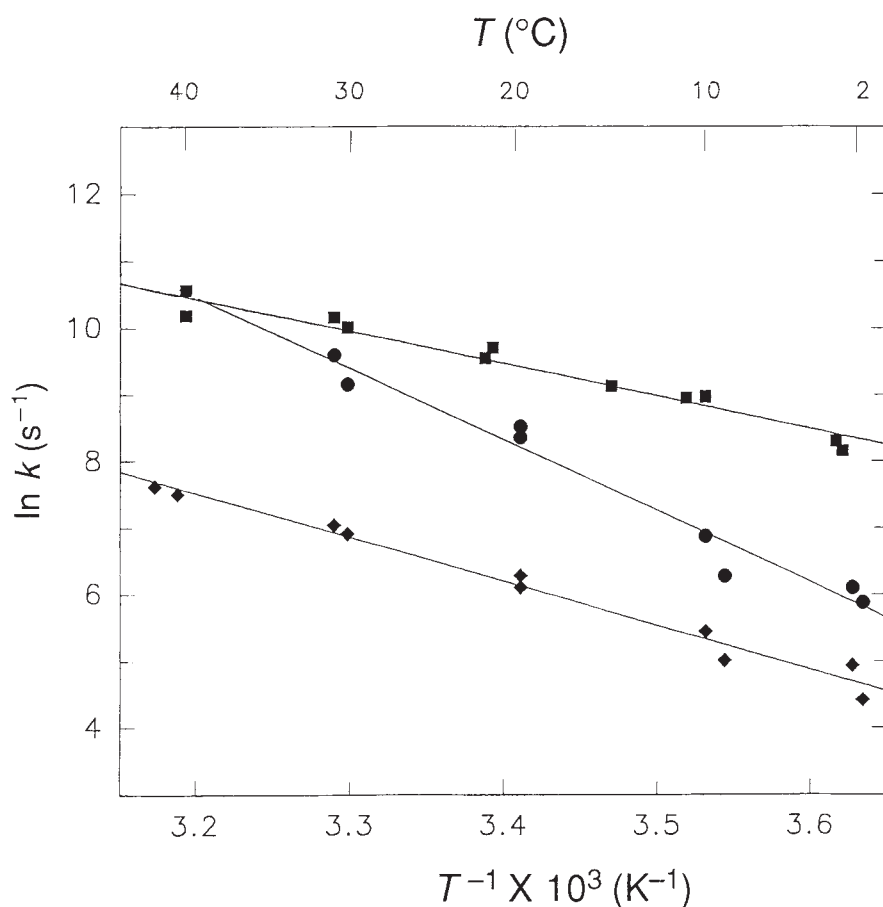


FIG. 3 Arrhenius diagram (logarithmic rate constants, k , versus reciprocal temperature) of the protonation reactions of fluorescein at Lys 129 at the ES of wild-type BR (■), fluorescein linked to Cys 36 at the CS (●), and pyranine residing in the aqueous bulk medium (◆). Activation energies: 40 kJ mol⁻¹ for intraproteinous proton transfer (■), 88 kJ mol⁻¹ for lateral proton transfer (●), and 54 kJ mol⁻¹ for surface to bulk transfer (◆).

brane suspension of $D_{app} = 3.4 \times 10^{-7}$ cm² s⁻¹ (which considers the buffering capacity of the purple membrane⁶), and the dimensions of the purple membrane, showed that protons being released at the extracellular surface arrive at the cytoplasmic surface within the same time as experimentally observed.

The surprisingly slow surface to bulk transfer rate, rather than an extraordinarily large diffusion coefficient, is the reason protons laterally diffuse macroscopic distances in the membrane interface compartment. In complex chemiosmotic systems, such as thylakoid and mitochondrial membranes, the kinetic competition between H⁺ equilibration with the bulk and efficient two-dimensional surface migration of H⁺ from a source to a sink, for example, a H⁺-ATP synthase, will be determined by various factors. Particularly important is the activity of the H⁺-delivering and consuming systems, their mutual distance, and the buffer capacity. If the rate of H⁺ consumption exceeds that of H⁺ generation, that is, if the membrane surface H⁺ capacity is not saturated, H⁺-surface transfer could significantly contribute to, or even dominate, chemiosmotic energy coupling. □

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Vitamin D₃–thyroid hormone receptor heterodimer polarity directs ligand sensitivity of transactivation

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THE nuclear receptors for 1,25-dihydroxyvitamin D₃ (VD) and 3,5,3'-triiodothyronine (T₃), that is, VDRs and T₃Rs respectively, control aspects of homeostasis, cell growth and differentiation^{1–4}. They activate transcription from response elements consisting of direct repeats, palindromes and inverted palindromes^{5–8} of a variety of hexameric core-binding motifs. VDRs bind preferentially to direct repeats spaced by three nucleotides, whereas T₃Rs bind to

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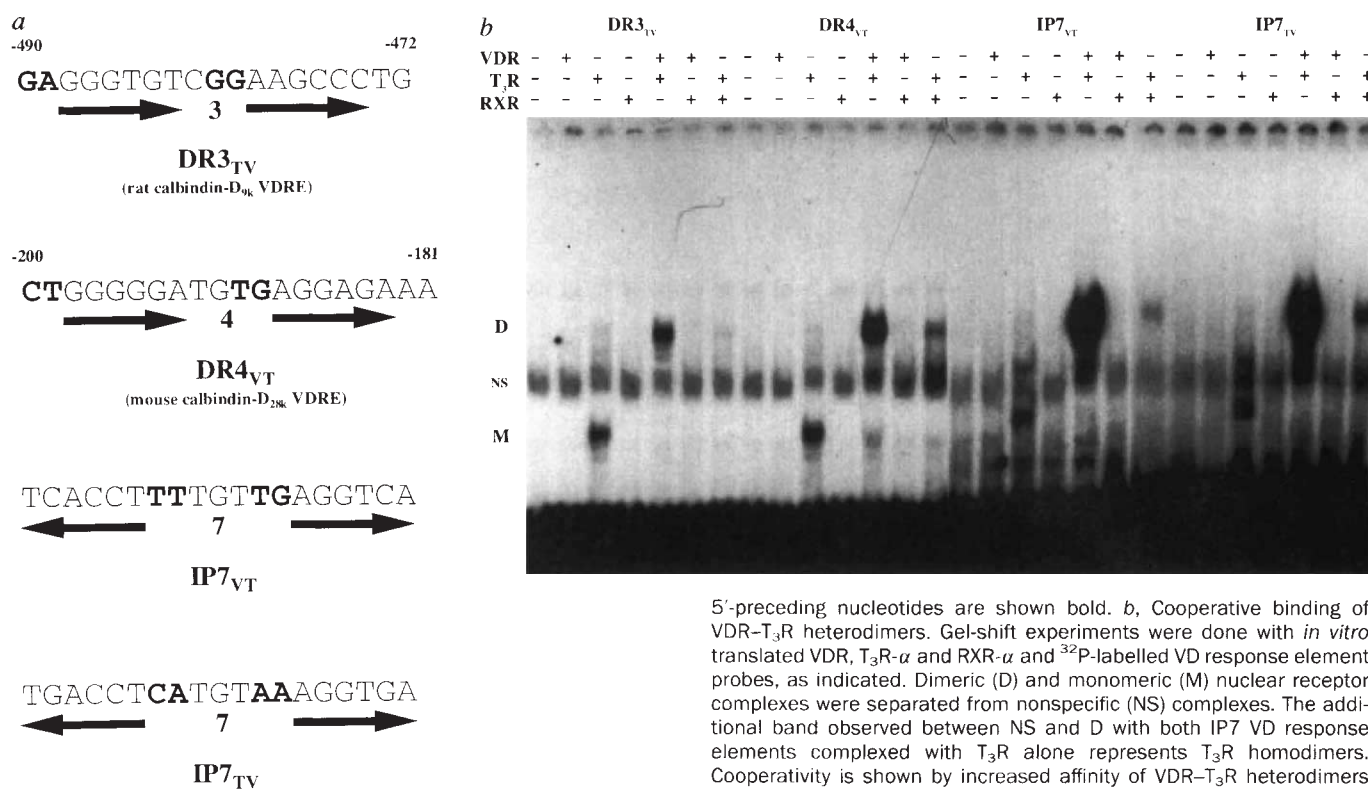


FIG. 1 Cooperativity of VDR-T₃R heterodimers. **a**, Response elements. Comparison of the binding affinity of VDR-VDR, VDR-RXR, VDR-RAR and VDR-T₃R complexes to all known natural VD response elements (VDRE) (ref. 17, and data not shown) showed that the VD response elements of rat calbindin-D_{9K} (ref. 18) and mouse calbindin-D_{28K} (ref. 19) are preferentially bound by VDR-T₃Rs. The sequences of these two natural VD response elements and of two inverted palindromic VD response elements (IP7_{VT} and IP7_{TV}), designed to be asymmetrical, are shown. Hexameric core-binding motifs are indicated by arrows and

direct repeats spaced by four nucleotides⁹. VDRs and T₃Rs can function as homodimers^{5,6,10} but heterodimerization with retinoid X¹¹⁻¹⁴ or retinoic acid receptors^{15,16} increases their affinity for DNA *in vitro* and resulting transcriptional activity *in vivo*. We recently observed the formation of VDR-T₃R heterodimers¹⁷. Here we show that the polarity of the binding of such heterodimers to the VD response element of the rat 9K (relative molecular mass 9,000) calbindin¹⁸ gene promoter was 5'-T₃R-VDR-3', whereas on the mouse 28K calbindin VD response element¹⁹ this polarity was reversed to 5'-VDR-T₃R-3'. We also show that the ligand for the downstream receptor controls the transcriptional activity of the heterodimeric complex. Thus, polarity seems to be an important regulatory property of heterodimeric nuclear receptor complexes.

The 28K calbindin VD response element is a direct repeat spaced by four nucleotides (DR4) and has a TG dinucleotide preceding the second core-binding motif (Fig. 1a); this suggests that a T₃R molecule should bind to this motif²⁰. However, the 9K calbindin VD response element is a direct repeat spaced by three nucleotides (DR3), on which, in the case of VDR-retinoid X receptor (RXR) heterodimers²¹, the VDR molecule binds to the downstream motif. By screening inverted palindromes with various motif sequences and spacers of 0-12 nucleotides, we found maximal affinity of VDR-T₃R heterodimers for inverted palindrome-7 (IP7; data not shown). In contrast to direct repeat motifs, response elements with an inverted palindrome structure are *per se* symmetrical. To introduce asymmetry, we designed IP7s with different core-binding motif sequences and 5'-preceding nucleotides (Fig. 1a), on which VDR and T₃R should each show clear preferences for one of the motifs.

Gel-shift experiments revealed cooperative binding of VDR-

5'-preceding nucleotides are shown bold. **b**, Cooperative binding of VDR-T₃R heterodimers. Gel-shift experiments were done with *in vitro* translated VDR, T₃R- α and RXR- α and ³²P-labelled VD response element probes, as indicated. Dimeric (D) and monomeric (M) nuclear receptor complexes were separated from nonspecific (NS) complexes. The additional band observed between NS and D with both IP7 VD response elements complexed with T₃R alone represents T₃R homodimers. Cooperativity is shown by increased affinity of VDR-T₃R heterodimers over VDR and T₃R homodimers and T₃R monomers. On all four response elements, the VDR-T₃R heterodimer complex is more prominent than VDR-RXR or T₃R-RXR heterodimer complexes.

METHODS. Pairs of oligonucleotides, each containing one of the four VD response elements with adjacent XbaI sites, were synthesized, purified, phosphorylated and annealed to yield double-stranded DNA fragments which were fused to the tk promoter to drive the expression of the CAT reporter gene by subcloning into the XbaI site of pBLCAT2 (ref. 29). *In vitro* translations of human VDR, chicken T₃R- α and human RXR- α and gel-shift experiments were as described¹⁷.

T₃R heterodimers to the four response elements DR3_{TV}, DR4_{VT}, IP7_{VT} and IP7_{TV} (Fig. 1b). Neither VDR nor RXR monomer or homodimer, nor VDR-RXR heterodimer binding, was observed. T₃Rs bound weakly as a monomer, a homodimer (in the case of IP7_{VT} and IP7_{TV}) or as a heterodimer with RXR. In contrast, VDR-T₃R heterodimers bound with high affinity to all four response elements. This affinity was not affected by the presence of VD or T₃ (alone or in combination) but was diminished by increasing amounts of RXR- α (Fig. 2a). To distinguish VDR and T₃R- α clearly by size, we truncated T₃R- α by 33 amino acids at the carboxy terminus by restriction of its complementary DNA with *Nar*I (producing Δ T₃R). Δ T₃R retains the ninth heptad repeat shown to be important for T₃R heterodimerization²² and formed a VDR- Δ T₃R heterodimer which showed higher mobility than VDR-T₃R (Fig. 2a). Using a crosslinking strategy similar to that of Zechel *et al.*²³, we investigated whether the differences in spacing, motif and flanking sequence between these four response elements indeed resulted in a polar heterodimeric binding of VDR and Δ T₃R. Figure 2b shows that Δ T₃R bound to the 5' motif of DR3_{TV} but to the 3' motif of DR4_{VT}, while VDR bound to the 5' motif of DR4_{VT} and to the 3' motif of DR3_{TV}. The same polarity was also observed on asymmetrical inverted palindromes. Δ T₃R bound to the 5' motif of IP7_{TV} but to the 3' motif of IP7_{VT}, whereas VDR bound to the 5' motif of IP7_{VT} and to the 3' motif of IP7_{TV}.

To investigate the significance of this polarity for transactivation by VDR and T₃R, we fused each of the four response elements to the heterologous thymidine kinase (*tk*) promoter/chloramphenicol acetyltransferase (CAT) reporter system,

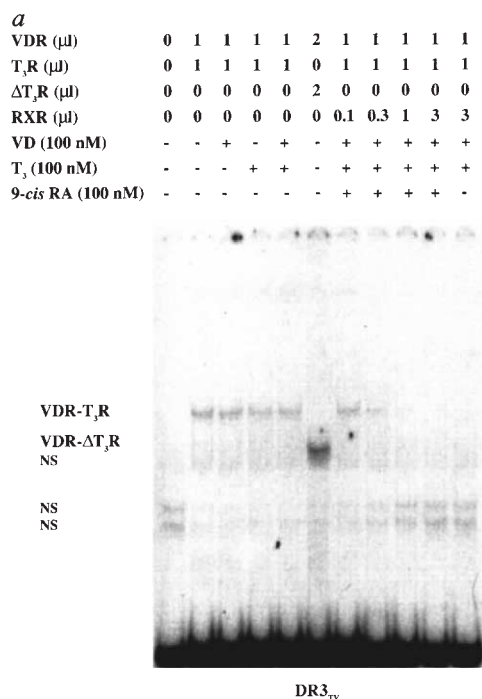
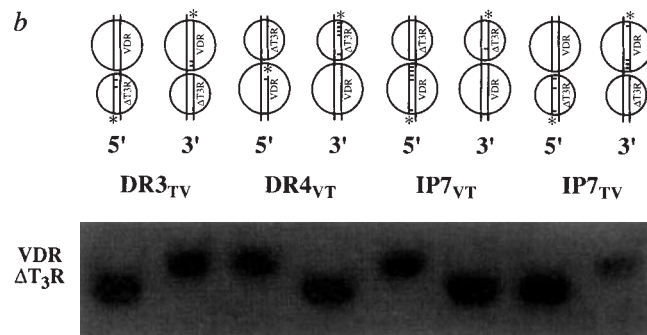


FIG. 2 Characterization of VDR-T₃R heterodimers. **a**, Gel-retardation analysis of VDR-T₃R heterodimers. Gel-shift experiments were carried out with *in vitro* translated VDR, T₃R- α , truncated T₃R- α (Δ T₃R) and RXR- α , their respective ligands or solvent and ³²P-labelled DR3_{TV} VD response element probe, as indicated. VDR-



T₃R heterodimer complex formation was not changed in the presence of VD and T₃, but diminished with increasing amounts of RXR. Δ T₃R formed a heterodimer with VDR which had a higher mobility than a heterodimer with full-length T₃R- α . **b**, Heterodimer polarity. VDR- Δ T₃R heterodimers were crosslinked to the 5' and the 3' motif of each of the four indicated response elements. The crosslinking strategy of Zechel *et al.*²³ using gapped, double-stranded oligonucleotides was applied and is depicted schematically. The radiolabelled oligonucleotides are indicated by asterisks and the thymidine residues substituted by 5-bromo-2'-deoxyuridine (BrUdr) are represented by bars. 5' and 3' represent the relative position of the indicated crosslinked receptor on the response elements.

METHODS. Gel-shift experiments with the indicated amounts of *in vitro* translated receptors were carried out as described²¹; ligands were added at the time of heterodimer formation. For the 5' and the 3' motif of each response element (see Fig. 1), BrUdr-substituted oligonucleotides were synthesized and phosphorylated with [γ -³²P]ATP. Each labelled oligonucleotide was annealed together with the unlabelled oligonucleotide for the second motif to the full-length complementary oligonucleotide to form a gapped double-stranded DNA fragment. Aliquots (10 μ l) of *in vitro* translated full-length VDR were incubated with equal amounts of Δ T₃R (1–375) and 1 ng of ³²P-labelled gapped probe (300,000 c.p.m.) in a final volume of 40 μ l binding buffer (Fig. 1) for 30 min at room temperature. UV crosslinking and electrophoresis were as described²².

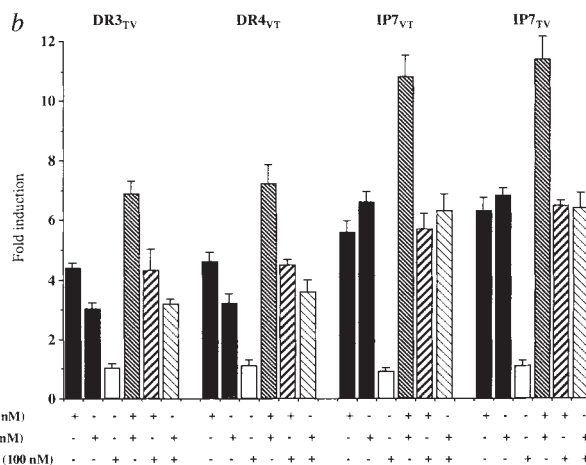
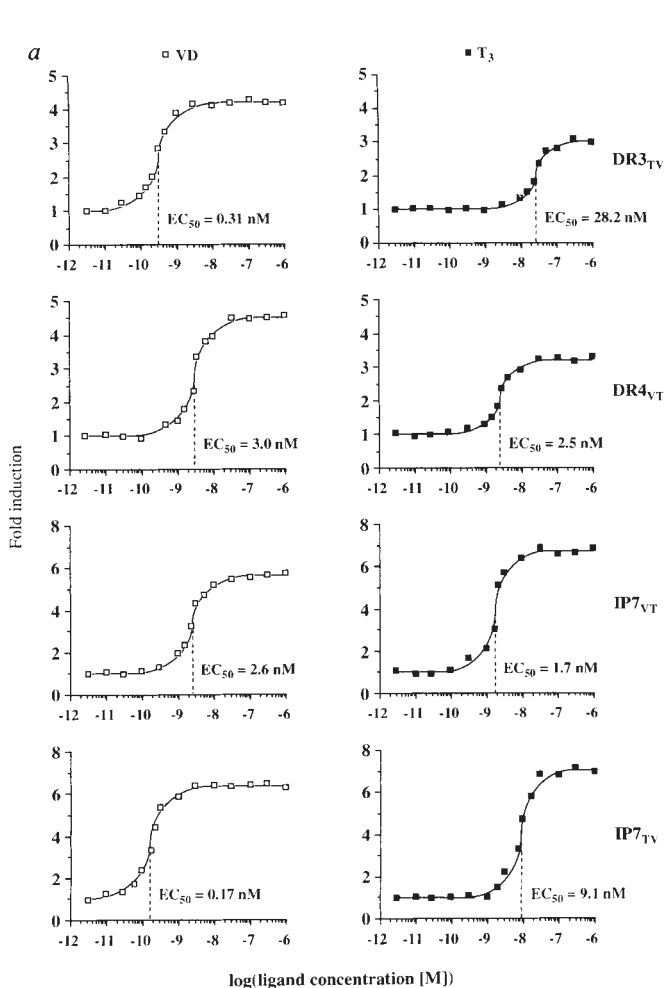


FIG. 3 Functional activity of VDR-T₃R heterodimers in a mammalian system. **a**, Dose responses to VD ($\square$) and T₃ ($\blacksquare$). Human MCF-7 cells were transfected with CAT reporter constructs containing the indicated VD response elements and treated with increasing concentrations of VD or T₃. CAT activities were determined 16 h later and stimulation was calculated compared with solvent-induced controls. Each data point represents the mean of triplicates; the standard deviation was always <10%. **b**, Co-stimulation with VD, T₃ and 9-cis RA. MCF-7 cells were transfected with CAT reporter constructs containing the indicated VD response elements. CAT activity was assayed 16 h after stimulation with VD, T₃ and 9-cis RA (each 100 nM), as indicated. Columns represent mean values of at least three independent experiments; the bars indicate standard deviations.

METHODS. MCF-7 (human breast cancer) cells (2×10^5 per well) were seeded into six-well plates and grown overnight in phenol-red-free RPMI (Life Technologies) supplemented with 10% charcoal-treated FCS. Liposomes were formed by incubating 2 μ g of the reporter plasmid and 1 μ g of the reference plasmid pCH110 (Pharmacia) with 15 μ g N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP; Boehringer Mannheim) for 15 min at room temperature in a total volume of 100 μ l. After dilution with 0.9 ml phenol-red-free RPMI the liposomes were added to the cells. 500 μ l phenol-red-free RPMI supplemented with 10% charcoal-treated FCS was added 4–8 h after transfection. At this time VD, 9-cis RA and T₃, combinations of them or solvent were also added, as indicated. The cells were collected 16 h after onset of the stimulation and CAT assays carried out as described³⁰. The CAT activities were normalized to β -galactosidase activity and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of mock-induced controls.

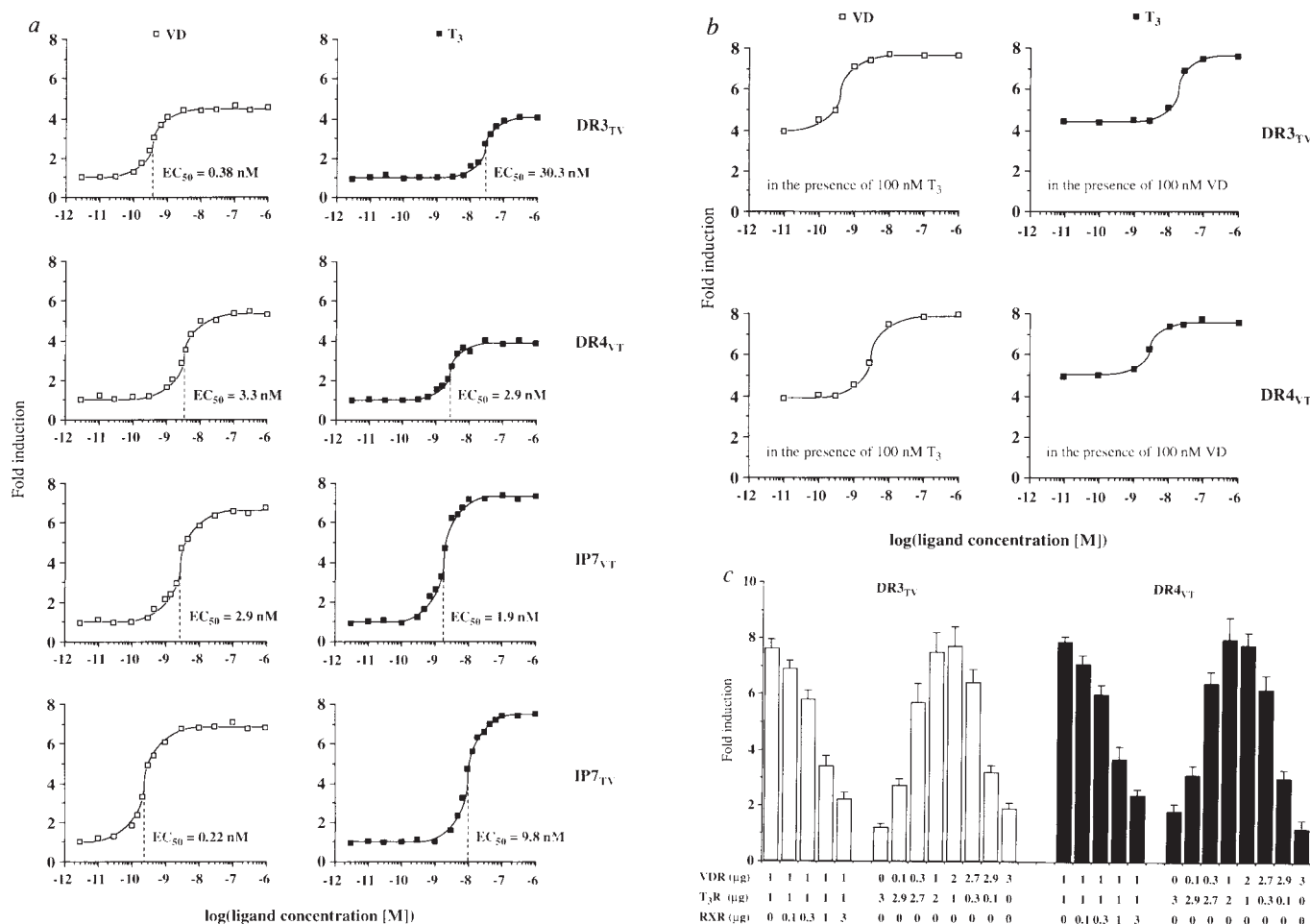


FIG. 4 Functional activity of VDR- T_3 R heterodimers in a *Drosophila* model system. **a**, Dose responses to VD (□) and T_3 (■). *Drosophila* SL-3 cells, which do not express nuclear receptors for VD, T_3 or retinoids, were transfected with expression vectors for VDR and T_3 R- α (1 μ g each) and CAT reporter constructs containing the indicated VD response elements. The cells were treated with graded concentrations of VD or T_3 , collected 16 h later and CAT activities determined as described for Fig. 3. **b**, Co-stimulation with VD (□) and T_3 (■). SL-3 cells were transfected with a combination of expression vectors for VDR and T_3 R- α (1 μ g each) and the CAT reporter constructs containing the natural VD response elements DR3_{TV} and DR4_{VT}. Dose responses to VD and T_3 were determined in the presence of saturating concentrations (100 nM) of the other ligand. **c**, Variation of receptor levels. SL-3 cells were transfected with the indicated amounts of expression vectors for VDR, T_3 R- α and RXR- α and CAT reporter constructs containing the VD response

transfected the constructs into human MCF-7 cells, and then stimulated the cells with graded concentrations of VD or T_3 (Fig. 3a). The resulting dose-response curves show that both ligands could stimulate gene activation by VDR- T_3 R heterodimers binding to all four response elements. However, the stimulations clearly differed in their 50% effective concentrations (EC_{50} s): on IP7_{TV} and DR3_{TV} the EC_{50} s for VD were 0.17 and 0.31 nM, respectively (of the order of magnitude determined for VDR-RXR heterodimers; C.C., unpublished results), and thus about 10-fold lower than for IP7_{VT} and DR4_{VT} (2.6 and 3.0 nM, respectively); conversely, the EC_{50} s for T_3 on IP7_{VT} and DR4_{VT} (1.7 and 2.5 nM, respectively) were clearly lower than on IP7_{TV} and DR3_{TV} (9.1 and 28.2 nM). Thus, half-maximal activation via VDR- T_3 R heterodimers was always more sensitive to VD when VDR bound to the 3' motif. The stimulation factors at saturating ligand concentrations, however, appeared to depend

elements DR3_{TV} or DR4_{VT}. CAT activity was assayed 16 h after stimulation with VD, T_3 and 9-*cis* RA (each 100 nM). Columns represent mean values of at least three independent experiments; bars indicate standard deviations.

METHODS. *Drosophila* SL-3 cells (5×10^5 cells per well in a 6-well plate) were grown overnight in Schneider's medium (Life Technologies) supplemented with 15% charcoal-treated FCS. Liposomes were formed by incubating 2 μ g of the reporter plasmid, the indicated amounts of pSG5-based VDR, T_3 R- α and RXR- α -expression vectors and 1 μ g of the reference plasmid pCH110 (Pharmacia) with 11 μ g DOTAP for 15 min at room temperature in a total volume of 100 μ l. After dilution with 0.9 ml Schneider's medium the liposomes were added to the cells; 4–8 h after transfection, 500 μ l Schneider's medium supplemented with 15% charcoal-treated FCS together with ligands or solvent were added.

only on the relative orientation of the core-binding motifs (direct repeat or inverted palindrome) and the ligand used, but not on the polarity of the heterodimeric complex. On all four response elements, stimulation with saturating concentrations of VD and T_3 in combination resulted in a stimulation which was less than the sum of the inductions with the ligands taken separately (Fig. 3b). MCF-7 cells do express RXR¹⁷; however, co-stimulation with its specific ligand 9-*cis* retinoic acid (RA) had no effect on VD and T_3 stimulation of any response element tested.

To demonstrate RXR-independent VDR- T_3 R activity, we analysed the dose responses to VD and T_3 in the *Drosophila* cell line SL-3 (Fig. 4a). As this cell line lacks mammalian nuclear receptors by definition, it represents a suitable model system for analysing the isolated activity of either homo- or heterodimers of VDR, RXR, RAR and T_3 R^{5–8,16,17,20}. SL-3 cells were transfected with equal amounts of expression vectors for VDR and

T₃R- α and with CAT reporter constructs and stimulated with VD and T₃. On all four response elements, the dose responses were very similar to those in MCF-7 cells (Fig. 3a), as the EC₅₀ values differed by less than 20%. On the two natural VD response elements DR_{3TV} and DR_{4VT}, we analysed the dose responses to VD and T₃ in the presence of saturating concentrations of the other ligand (Fig. 4b). The latter mediated an upshift of the dose-response curve, but did not affect the EC₅₀. In the SL-3 model also, maximal stimulation with saturating concentrations of both ligands was less than the sum of the effects of VD and T₃ alone. This suggests non-synergistic stimulation of VDR-T₃R heterodimers by VD and T₃.

Figure 4c shows that hormone responsiveness was maximal when VDR and T₃R- α concentrations were about the same, suggesting that the functional activity of VDR-T₃R heterodimers depends on the VDR:T₃R ratio. Consequently, co-expression of increasing amounts of RXR- α in SL-3 cells resulted in decreasing functional activity of VDR-T₃R heterodimers, either in the presence of 100 nM 9-*cis* RA (Fig. 4c) or in its absence (data not shown). Retinoids therefore appear not to affect the functional activity of VDR-T₃R heterodimers. Although RXR overexpression in SL-3 cells confirmed the squelching effect we observed *in vitro* (Fig. 2a), the complexity of nuclear receptor signalling in mammalian systems may prevent RXR from significantly affecting the combined activity of VDR and T₃R in MCF-7 cells (Fig. 3b).

The binding polarity of heterodimers formed by RXR with RAR or T₃R on direct repeats spaced by two, four and five nucleotides has recently been demonstrated^{21,24-26}. We show here that polar binding of VDR-T₃R heterodimers occurs not only on direct repeats, but also on inverted palindromes consisting of asymmetrical core-binding motifs and 5'-preceding nucleotides. This suggests that natural hormone response elements with an inverted palindrome structure should be as functional as direct repeats. Distinct transcriptional outcomes have already been predicted as a consequence of the polarity of RXR-RAR and RXR-T₃R heterodimers^{23,25-27}. Therefore, a polarity-dependent ligand sensitivity, as reported here for VDR-T₃R heterodimers, may also direct the activity of other heterodimeric complexes. Our observation suggests that low ligand concentrations are sufficient for transactivation by heterodimers through the 3' receptor, whereas the 5' receptor stimulates transactivation only at high concentrations of its ligand. The fact of whether the tissue-specific intracellular ligand concentration is above or below this threshold should determine the role of this ligand in controlling transactivation. For instance, VD-dependent 28K calbindin expression is observed in various tissues, but not in the brain²⁸; we would therefore expect cerebral intracellular VD concentrations to be below this threshold. □

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The ETS domain protein Pointed-P2 is a target of MAP kinase in the Sevenless signal transduction pathway

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THE fate of the R7 photoreceptor cell in the developing eye of *Drosophila* is controlled by the Sevenless (Sev) receptor tyrosine kinase^{1,2}. Sev activates a highly conserved signal transduction cascade involving the proteins Ras1 and Raf and the Rolled/mitogen-activated protein (R1/MAP) kinase³. Here we show that the ETS domain protein encoded by the P2 transcript of the *pointed* (*pnt*) gene is a nuclear target of this signalling cascade which acts downstream of R1/MAP kinase. The PntP2 protein is phosphorylated by R1/MAP kinase *in vitro* at a single site and this site is required for its function *in vivo*. Furthermore, we present genetic and biochemical data suggesting that MAP kinase controls neural development through phosphorylation of two antagonizing transcription factors of the ETS family, Yan and PntP2.

Genetic studies have suggested that activation of the MAP kinase encoded by the *rolled* (*rl*) gene is a critical event in the signal transduction process specifying the R7 photoreceptor fate during *Drosophila* eye development^{4,5}. Persistent activation causes vertebrate MAP kinase to translocate into the nucleus and to phosphorylate transcription factors such as the ETS protein Elk-1 (refs 6-8). Phosphorylation of Elk-1 is necessary for its function as a transcriptional activator⁹. In *Drosophila* the genes *yan* and *pnt* both encode members of the ETS family of proteins (Fig. 1a)¹⁰⁻¹². We tested whether these ETS proteins are targets of Sev-signalling-dependent R1/MAP kinase activation.

Like *yan*¹¹, *pnt* is expressed in undifferentiated cells of the eye imaginal disk (Fig. 1b, c). The PntP2 protein accumulates in the nuclei of all ommatidial precursor cells. In the photoreceptor precursors, PntP2 expression precedes the appearance of the neuronal marker Elav and is no longer detected in these cells shortly after they begin to express Elav (Fig. 1e). Therefore, PntP2 is expressed in ommatidial cells at the same time as Sev signalling is known to control cell fate specification¹³. Mutations that reduce PntP2 function affect the specification of R7 photoreceptor cells. In flies carrying the viable allelic combination of *pnt*¹²⁷⁷/*pnt*^{Δ88}, most ommatidia lack one to three photoreceptor cells (Fig. 2b). This phenotype is reminiscent of that found in

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These data suggest that PntP2 may be a direct target for RI/MAP kinase, as has been shown for ETS proteins in vertebrates^{9,18}. Indeed, the P2 protein contains a single MAP kinase phosphorylation site (PLTP) motif in the Pointed domain corresponding to the consensus for MAP kinase phosphorylation¹⁹ and can be phosphorylated by RI/MAP kinase *in vitro* (Fig. 3a, lane 2). Phosphorylation is dependent on this single site because a mutant protein (PntP2^{T151A}) in which the threonine in the PLTP motif has been replaced by an alanine cannot be phosphorylated *in vitro* (Fig. 3a, lane 4). To test whether this mutation affects the function of PntP2 *in vivo*, we generated transgenic lines expressing the mutant transgene under the control of the *sev* enhancer (*sE-pnt^{T151A}*). Not only is this construct unable to rescue the *pnt* phenotype, rather it enhances the mutant phenotype (Fig. 2g). Even in a wild-type background the mutant protein prevents neural development of the R7 precursor (Fig. 1i) which results in the absence of R7 cells in the adult (Fig. 2h). This suggests that the mutant protein competes directly or indirectly with the wild-type protein. Similar to Pnt^{T151A}, a mutant form of vertebrate Elk-1 in which multiple MAP kinase phosphorylation sites have been deleted prevents serum response element-dependent transcription in a dominant-negative fashion⁹.

Loss-of-function mutations in *yan*, which encodes another ETS domain protein, result in the recruitment of many R7 cells even in the absence of *sev* function¹¹ (Fig. 2i). In *yan*, *pnt* double mutants most of the ommatidia lack R7 and some outer photoreceptors (Fig. 2k), suggesting that the development of R7 cells in the absence of *yan* function depends on *pntP2* function. Although overexpression of *pntP2* under the control of the *sev* enhancer is not sufficient to transform cone cells into R7 cells in the wild type (Fig. 2l), many R7 cells form in a heterozygous *yan* background (Fig. 2m). These observations suggest that the mechanisms controlling R7 determination are sensitive to changes in the relative amounts of PntP2 as well as of Yan. The Yan protein contains 10 consensus sites for MAP kinase phosphorylation¹⁹ and, like PntP2, can be phosphorylated by MAP kinase *in vitro* (Fig. 3a, lane 6).

Our data thus suggest a model in which MAP kinase activity induces neuronal differentiation by simultaneously inhibiting the Yan repressor and stimulating the PntP2 activator (Fig. 3b). The fact that both proteins contain an ETS DNA-binding domain known to recognize conserved target sequences²⁰ raises the possibility that these two proteins compete directly for binding sites present in the regulatory regions of target genes. □

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Rapid evolution of a protein *in vitro* by DNA shuffling

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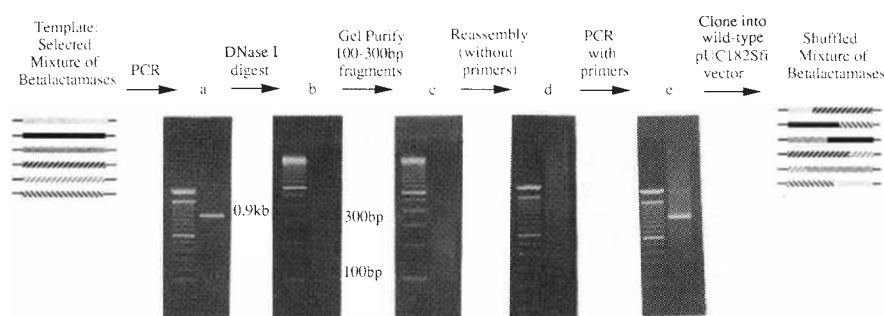
DNA SHUFFLING is a method for *in vitro* homologous recombination of pools of selected mutant genes by random fragmentation and polymerase chain reaction (PCR) reassembly¹. Computer simulations called genetic algorithms^{2–4} have demonstrated the importance of iterative homologous recombination for sequence evolution. Oligonucleotide cassette mutagenesis^{5–11} and error-prone PCR^{12,13} are not combinatorial and thus are limited in searching sequence space^{1,14}. We have tested mutagenic DNA shuffling for molecular evolution^{14–18} in a β -lactamase model system^{9,19}. Three cycles of shuffling and two cycles of backcrossing with wild-type DNA, to eliminate non-essential mutations, were each followed by selection on increasing concentrations of the antibiotic cefotaxime. We report here that selected mutants had a minimum inhibitory concentration of 640 $\mu\text{g ml}^{-1}$, a 32,000-fold increase and 64-fold greater than any published TEM-1 derived enzyme. Cassette mutagenesis and error-prone PCR resulted in only a 16-fold increase⁹.

The poorly hydrolysed antibiotic cefotaxime has a minimum inhibitory concentration (MIC) of only 0.02 $\mu\text{g ml}^{-1}$ for *Escherichia coli* containing the TEM-1 β -lactamase expressed from the vector p182Sfi. The TEM-1 gene was digested into random fragments with DNase I. These small fragments were reassembled into full-length sequences using a PCR-like process and the shuffled sequences reinserted into the vector¹ (Fig. 1). Recombination is caused by the incorporation of a fragment derived from one sequence into another, based on homology. In addition, this method produces a point mutagenesis rate of 0.7%, similar to error-prone PCR^{1,13}. This process was repeated for three rounds, and after each round, mutants with improved resistance were selected by plating on increasing levels of cefotaxime. Several hundred colonies from the highest levels of cefotaxime were used as the PCR template for the next round (Fig. 2). Colonies from rounds 1, 2 and 3 were obtained at 0.32–0.64 $\mu\text{g ml}^{-1}$, 5–10 $\mu\text{g ml}^{-1}$ and 40–80 $\mu\text{g ml}^{-1}$, respectively. Some colonies from round 3 had a MIC of 320 $\mu\text{g ml}^{-1}$. Because cefotaxime resistance is cell-density-dependent, the MIC was standardized to 1,000 cells per plate (24 h, 37 °C). At higher cell density, colonies grew at up to 1,280 $\mu\text{g ml}^{-1}$. A β -lactamase gene (ST-1) of a selected colony contained nine base substitutions, including four silent mutations (Fig. 2).

We attempted to remove all non-essential mutations by backcrossing¹. ST-1 was shuffled for two rounds in the presence of a 40-fold excess of wild-type DNA fragments (Fig. 2). Small DNA fragments (30–100 bp) were used to increase the efficiency of the backcross. A new β -lactamase gene (ST-2), obtained at 1,280 $\mu\text{g ml}^{-1}$, had a MIC of 640 $\mu\text{g ml}^{-1}$. As expected, all four silent mutations had reverted to wild-type sequence. Mutation g4205a, located between the –35 and –10 sites of the β -lactamase P3 promoter, was retained. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of periplasmic extracts showed that ST-1 and ST-2 express 2–3-

FIG. 1 DNA shuffling of the TEM-1 β -lactamase followed by selection for cefotaxime resistance. A single round of shuffling consists of amplifying the β -lactamase gene by PCR, cutting the PCR product into random fragments with DNase I, gel purification of small fragments, reassembly of the fragments in a PCR-like reaction without primers, amplification of the reassembled product by standard PCR, followed by cloning into the vector and selection on cefotaxime.

METHODS. p182Sfi contains the TEM-1 β -lactamase flanked by *Sfi*I restriction sites. The *Sfi*I sites were added 5' of the promoter and 3' of the end of the gene by PCR of the vector with the primers TTCTATTGACGGCCTGTACAGCCTCAT-ATATACTTTA-GATTGATTT and TTGACGCACTGGCCATGGTGGCCAAAAATAACAAAT-AGGGGTTCCGCGCACATTT, and by PCR of β -lactamase gene with two other primers listed below. The substrate was used as the template for the PCR. Colony PCR programme: 10 μ l of cells in LB broth, 10 min, 99 °C, 35 $\times$ (94 °C, 30 s; 52 °C, 30 s; 72 °C, 30 s), 5 min, 72 °C. The removal of free primers from the PCR product by Wizard PCR prep (Promega, Madison, WI) was found to be very important. A few μ g of the DNA



substrate was digested with 0.15 units of DNase I (Sigma) in 100 μ l 50 mM Tris-HCl pH 7.4, 1 mM MgCl₂, for 10 min at room temperature. Fragments of 100–300 bp were purified from a 2% low melting point agarose gel and resuspended in PCR mix (0.2 mM each dNTP, 2.2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100) at 10–30 ng μ l⁻¹. No primers were added at this point. A PCR programme of 94 °C, 1 min, 40 $\times$ (94 °C, 30 s; 50–55 °C, 30 s; 72 °C, 30 s), was used in an MJ Research PTC-150 (Watertown, MA) thermocycler. After 40-fold dilution of the minus primer product into PCR mix with 0.8 μ M of each primer and 15–20 additional cycles of PCR (94 °C, 30 s; 50 °C, 30 s; 72 °C, 45 s), reproducibly a single product of 900 bp is obtained.

TABLE 1 Characterization of cefotaxime resistance of different combinations of mutations

Name	Genotype	MIC	Source of MIC
TEM-1	Wild-type	0.02	This study
—	E104K	0.08	Ref. 9
—	G238S	0.16	Ref. 9
TEM-15	E104K/G238S*	10	This study
TEM-3	E104K/G238S/Q39K	10*	This study
		2–32	Refs 19, 20
ST-4	E104K/G238S/M182T*	10	This study
ST-1	E104K/G238S/M182T/ A18V/t3959a/g3713a/ g3934a/a3689g*	320	This study
ST-2	E104K/G238S/M182T/ A42G/G92S/R241H/ t3842c/a3767g*	640	This study
ST-3	E104K/G238S/M182T/ A42G/G92S/R241H*	640	This study



The base numbers (small letters) correspond to the revised pBR322 sequence²⁴, and the amino-acid numbers (capitals) correspond to the ABL standard numbering scheme²⁵. Specific combinations of mutations were introduced into the wild-type p182Sfi by PCR, using two oligonucleotides per mutation. The separate PCR fragments were gel purified and combined by overlap PCR using 10 ng of each fragment. PCR was done for 25 cycles without outside primers, followed by 25 cycles in the presence of the *Sfi*I-containing outside primers. The oligonucleotides for mutation A42G were AGTTGGGTGGACGAGTGGGTTACATCGAACT and AACCCACTCGTCCACCACTGATCTTCAGCAT, for Q39K AGTAAAGAT-GCTGAAGATAAGTTGGGTGCACGAGTGGGTT and ACTTATCTTCAGCATC-TTTTACTT, for G92S AAGAGCAACTCAGTCGCCGCATACACTATCT and AT-GCGGCGACTGAGTTGCTCTTGCCCGCGCTCAAT, for E104K TATTCTCAG-AATGACTTGGTTAAGTACTACCACTGACATGCAAT and TTAACCAAGTCACTT-GAGAAT, for M182T AACGACGAGCGTGACACACGACGCTGTAGCAAT-GGCAA and TCGTGGTGTACGCTCGTCTGCT, for G238 alone TTGCTGATAAATCTGGAGCCAGTGAGCGTGGGTCTCGCGGTA and IGGCT-CAGATTATATCAGCAAT, for G238S and R241H (combined) ATGCTCA-CTGGCTCCAGATTATCAGCAAT and TCTGGAGCCAGTGAGCATGGGTCTC-GCGGTATCATT, for g4205a AACCTGTCTCGCCACCATGGCCATAATACAA-TCAAATATGTATCCGCTTATGAGACAATAACCTGATA.

* All these mutants additionally contain the g4205a promoter mutation.

fold more β -lactamase than the wild-type plasmid (data not shown). ST-2 contained three of the four amino-acid mutations of ST-1 (E104K, M182T and G238S), as well as three new amino-acid mutations (c3441t resulting in R241H, c3886t resulting in G92S, and g4035c resulting in A42G) and two new silent mutations (t3842c and a3767g).

For comparison with published data, several combinations of mutations were constructed into the wild-type p182Sfi vector (Table 1). All contained the g4205a promoter mutation, and were confirmed by partial sequencing. The known clinical TEM-1-derivatives (TEM-1–19) all contain up to four of a set of eight dispersed mutations^{19–21}. Because the maximum MIC obtained by cassette mutagenesis was only 0.64 μ g ml⁻¹ (ref. 9), high resistance apparently cannot be obtained by mutagenesis of one area. Mutations E104K or G238S are present in published cefotaxime-resistant TEM-1 derivatives (TEM-3, 4, 6, 8, 9, 14–19), and were obtained separately by cassette mutagenesis, with MICs of only 0.08 and 0.16 μ g ml⁻¹ (ref. 9). In contrast, a combinatorial mutagenesis approach might have yielded the double mutant (TEM-15; ref. 19) with a MIC of 10 μ g ml⁻¹. These two mutations thus appear to be synergistic. E104K and G238S in combination with Q39K (TEM-3) or T263M (TEM-4) have reported MICs of 2–32 μ g ml⁻¹ (refs 19, 21), whereas a TEM-3 like construct in our vector had a MIC of 10 μ g ml⁻¹. A construct containing the three amino-acid changes that were conserved after the backcross (E104K, M182T, G238S) also had a MIC of 10 μ g ml⁻¹. With or without the silent mutations, constructs containing all of the six amino-acid changes of ST-2 introduced into the wild-type gene yielded colonies with the same MIC as ST-2 (640 μ g ml⁻¹). Thus, the six amino-acid mutations (plus the promoter mutation) conferred the high-resistance phenotype.

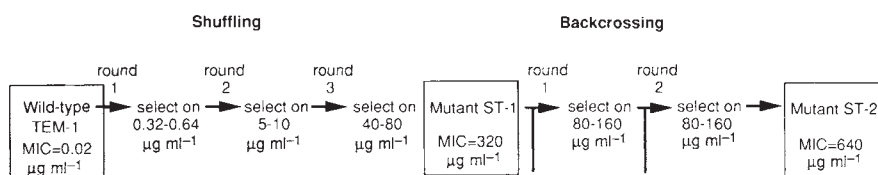
A control experiment using error-prone PCR but no shuffling resulted in a MIC of only 0.32 μ g ml⁻¹ after three selection cycles.

A useful approach may be to shuffle many related, naturally occurring genes, such as antibodies^{22,23} or homologous genes from different species. The diversity present in such a mixture may be more meaningful than random mutations. □

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FIG. 2 Three successive rounds of DNA shuffling were done and the cells were grown on increasing cefotaxime levels. The MIC of cefotaxime (Sigma) for *E. coli* XL1-blue (Stratagene, San Diego) carrying wild-type p182Sfi is $0.02 \mu\text{g ml}^{-1}$. A mutant with a 16,000-fold increased resistance to cefotaxime was obtained ($\text{MIC} = 320 \mu\text{g ml}^{-1}$). This mutant was backcrossed twice, by shuffling with a 40-fold excess of wild-type DNA. The backcrossed mutant was 32,000-fold more resistant than the wild type ($\text{MIC} = 640 \mu\text{g ml}^{-1}$). After selection, the plasmid of selected clones was transferred back into wild-type XL-1 blue cells to ensure that none of the measured drug resistance



was due to chromosomal mutations. DNA sequencing showed that both mutants had 9 single-base-pair mutations.

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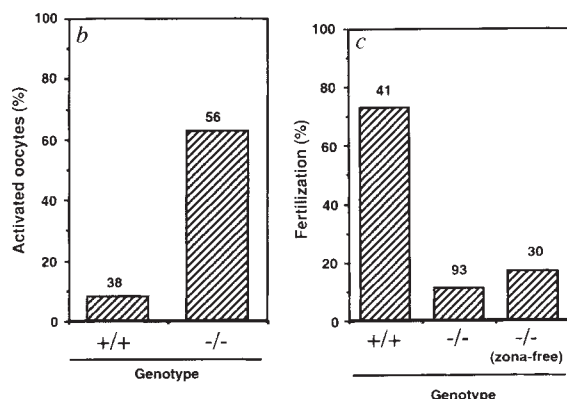
ERRATA

Parthenogenetic activation of oocytes in *c-mos*-deficient mice

Naohiro Hashimoto, Nobumoto Watanabe, Yasuhide Furuta, Hiroyuki Tamemoto, Noriyuki Sagata, Minesuke Yokoyama, Kenji Okazaki, Mariko Nagayoshi, Naoki Takeda, Yoji Ikawa & Shinichi Aizawa

Nature **370**, 68–71 (1994)

FIGURE 3*b* and *c* of this Letter was an early version that should not have been published. The correct version of this figure is shown here. □



Degradation of trifluoroacetate in oxic and anoxic sediments

Pieter T. Visscher, Charles W. Culbertson & Ronald S. Oremland

Nature **369**, 729–731 (1994)

In the last sentence of the opening paragraph of this Letter, an error was introduced during editing in which fluoroform was referred to as a “potential ozone-depleting compound.” In fact, fluoroform as well as other HFCs were recently shown by Ravishankara *et al.*¹ to have “negligibly small” ozone depletion potentials. □

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Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase

Mitsuharu Hattori, Hideki Adachi, Masafumi Tsujimoto, Hiroyuki Arai & Keizo Inoue

Nature **370**, 216–218 (1994)

THE word ‘acetylhydrolase’ was accidentally omitted from the end of the title of this paper. The correct title should read “Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase”. □

Communication by helix

Understanding the complex web of intracellular signalling pathways is brought a step closer by determinations of the structure of the SH3 domain/peptide ligand interface.

THE cells that make up a multicellular organism are able to sense and respond to a vast number of signals in their environment. These signals may direct them to proliferate, alter their architecture or their metabolism, differentiate or even die. This barrage of information must be integrated and acted upon so that the cell, and as a consequence the whole organism, can continue functioning normally. How is this maze of biochemical pathways coordinated? The answer, at least for a small part of the network, seems to reside in two small independently folded protein modules, the Src-homology domains SH2 and SH3. These motifs — found in a wide range of proteins involved in signalling and, for SH3, in the cytoskeleton — are suggested to act as molecular adaptors linking and regulating the location and/or activity of molecules such as tyrosine kinases and small GTP-binding proteins. The nature of the interaction between either the SH2 and/or SH3 modules and their protein ligands — which may themselves contain SH2 and SH3 domains — is likely to be critical in determining the fate of the signal and the subsequent action taken by the cell. The first glimpse of an SH3/peptide ligand interface is presented in this month's *Nature Structural Biology*¹ and in a recent paper published in *Cell*².

A number of protein ligands that interact with various SH3 domains have been characterized and the binding site in each case has been localized to a sequence rich in proline residues. Similar results have been obtained from screens of combinatorial peptide libraries. At the same time several structures of SH3 domains in the absence of ligand have been determined³, and these reveal that the core of the domain is formed by two three-stranded β -sheets which are perpendicular to one another. The most highly conserved residues form a hydrophobic platform

nestling between two loops of variable length that connect strands in the sheets. The conservation of the hydrophobic residues in the platform together with NMR experiments have suggested that this pocket is the ligand binding site, a suggestion that is now confirmed^{1,2}.

Musacchio and colleagues have determined the X-ray crystal structures of the SH3 domains of the tyrosine kinases Abl and Fyn, either alone or complexed with proline-rich peptide from the protein ligands 3BP-1 and 3BP-2, respectively. The two different bound peptides adopt very similar conformations, the majority of the residues adopting a left-handed polyproline II helix (PPII) conformation, as is seen in the NMR structure of the phosphatidylinositol-3-OH kinase SH3 domain bound to peptide selected from a combinatorial library². PPII helices are ideally suited as a target for SH3 domains. Because of their lack of internal hydrogen bonding and dependence on backbone solvation for stability, they are, for the most part, found on the surfaces of proteins making them effective substrates for protein-protein-mediated interactions.

No dramatic conformational changes are seen in the structures of any of the SH3 domains on binding ligand, suggesting that shape complementarity is important for the interaction between the protein motifs and the proline-rich peptides. The PPII helix, almost twice as long as an α -helix with the same number of residues, fits comfortably into the long and shallow groove of the SH3 binding pocket. The PPII helix has three residues per turn; every fourth residue (that is, residues i and $i+3$) sticks out from the peptide in roughly the same direction to form three groups of residues with similarly orientated side chains. Two of these groups of residues, all prolines in the case of 3BP-1 (i and $i+1$; $i+3$ and $i+4$), and equivalent residues in the NMR structure, interdigitate with two of the conserved aromatic residues in the ligand-binding site⁴. The prolines i , $i+3$ are known from mutagenesis studies to be particularly important for binding. Indeed comparison of the sequences of known SH3 binding motifs reveals that the PXXP (i , $i+3$) motif is found in all cases.

To avoid hopelessly tangling up the signal transduction network in the cell, it is important that signalling molecules and ancillary factors are recruited to the right proteins on the pathway. The van der

Waals interactions involving the conserved proline residues are seen in all the complexes, and although these interactions are likely to provide most of the binding energy for complex formation, they provide little specificity of binding. Musacchio *et al.* note that the equivalent hydrogen bonds in the Abl and Fyn SH3 complexes are directed to the main-chain atoms of the peptides and are therefore not specific for the peptide sequence. However, these hydrogen bonds may be important in selecting the orientation of binding of the ligand.

The two studies come to somewhat different conclusions as to what is likely to dictate the specificity and therefore the effector function of the many SH3 domains in signalling and cytoskeletal proteins. Yu *et al.* suggest that non-proline residues on the two faces of the PPII helix that can interact with the SH3 domain may be able to make complementary interactions with the more variable residues in the vicinity of the ligand-binding site, and particularly with residues in the two variable loops that border the hydrophobic platform. For example, they observe two salt bridges between the first and sixth residues of the ligand and these loops. Mutation of the residues in SH3 and disruption of the salt bridge result in a loss of binding affinity, by fiftyfold in one case². On the other hand, Musacchio *et al.* detect no non-proline interactions in the Abl or Fyn SH3 complexes. They point out that the SH3 domain may not be a functionally independent unit but may need to interact with SH2 or other SH3 domains to be fully functional. Perhaps there are still other factors to be taken into account in the analysis of these adaptor proteins: what, for example, is the role of another domain, the pleckstrin-homology domain⁵, which also occurs in the same families of proteins, and how does this motif influence intracellular signalling?

Guy Riddihough

Guy Riddihough is Editor of Nature Structural Biology.

Also in this month's *Nature Structural Biology*: site-directed isotope labelling and Fourier-transform infrared difference spectroscopy; dissecting protein stability in Arc repressor; a reaction mechanism for haem peroxidases; Clara cell phospholipid-binding protein with ligand; tunnelling through bromoperoxidase; a tethered dimer of HIV proteinase with inhibitor; glycoprotein dynamics; protein splicing; and atypical mitochondrial transfer RNAs.

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High-resolution mass spectrometer for protein chemistry

Yunzhi Li, Richard L. Hunter and Robert T. McIver Jr

Matrix-assisted laser desorption/ionization is an accurate and sensitive method for measuring the molecular weights of peptides and proteins. Usually time-of-flight mass spectrometry is used to detect the laser-produced ions, but a new method called Fourier transform mass spectrometry offers greater sensitivity and much higher mass measurement accuracy.

In 1987, a revolutionary new mass spectrometry method called matrix-assisted laser desorption/ionization (MALDI) was discovered for measuring the molecular weights of biopolymers¹⁻⁴. The key idea is to isolate and surround the analyte biomolecules with a matrix material that strongly absorbs laser light. When a laser pulse strikes the solid matrix/biomolecule mixture, ablation and ionization of the surface layer occurs very rapidly. One might expect that fragile biomolecules would not survive such a violent event, but remarkably they remain intact and appear as the main peaks in a mass spectrum. Time-of-flight mass spectrometry (TOF-MS) was used for the first MALDI experiments because of its unlimited mass range, simplicity and the ease with which pulsed ion signals could be detected. With the introduction a few years ago of inexpensive MALDI-TOF instruments, the technique has become an essential tool for research, development and quality control applications in many protein chemistry laboratories⁵⁻¹¹.

The main limitation of TOF for biomolecule analysis is the relatively low mass resolution. Linear TOF instruments typically have a resolution of 500, and with reflectron designs the resolution can be improved to several thousand¹²⁻¹⁶. Recently, we described how Fourier transform mass spectrometry (FTMS) could be used with MALDI to achieve much higher mass resolution¹⁷. Experiments have shown that this method provides the highest mass resolution ever reported for MALDI-produced ions, up to 90,000 for bovine insulin and 1,100,000 for small peptides¹⁸⁻²⁰. This enables the ¹³C-isotope peaks of a molecule to be readily distinguished and greatly improves the accuracy of molecular weight measurements.

In this article, the principles of FTMS are briefly described, examples of high mass resolution are presented and the relative advantages of MALDI-FTMS are discussed.

FT mass spectrometer

In most mass spectrometers, ions representative of the analyte are detected by the electrical current they produce when they crash into the surface of a device such as an electron multiplier. Although this method is widely used and very sensitive, it has the disadvantage that the ions must be destroyed in order to be detected. In FTMS the detection method is fundamentally different: a strong magnetic field traps ions inside an analyser cell and electrical signals produced by their cyclotron motion are detected by a pair of metal electrodes connected to a high impedance amplifier²¹⁻²⁴. This image current detection method 'senses' the number of ions without removing them from the analyser cell and without destroying them. This is similar to the way that magnetic resonance (nuclear magnetic resonance and magnetic resonance imaging) signals are detected. With FTMS a few hundred ions are usually needed to produce a useful signal, but with care the image current method can detect a single ion^{25,26}.

Figure 1 shows a schematic drawing of the prototype FTMS instrument that has been built at the University of California, Irvine. There are four basic parts: a laser for ionizing the sample, an evacuated stainless steel chamber, a superconducting magnet and a data system (not shown) for controlling the instrument and acquiring mass spectra. Samples are prepared for MALDI analysis by dissolving them in 30% acetonitrile/water and adding the matrix solution, 2,5-dihydroxybenzoic acid (DHB) in ethanol²⁷.

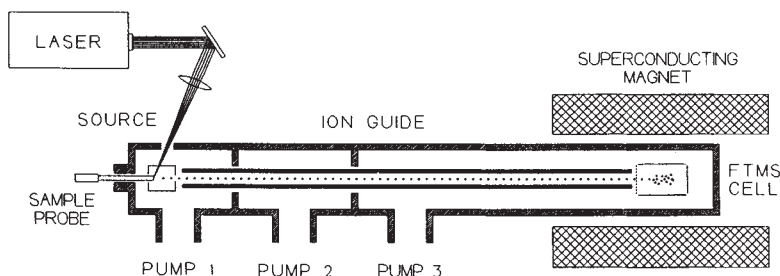


FIG. 1 Fourier transform mass spectrometer with an external ion source and a laser for ionizing biomolecules.

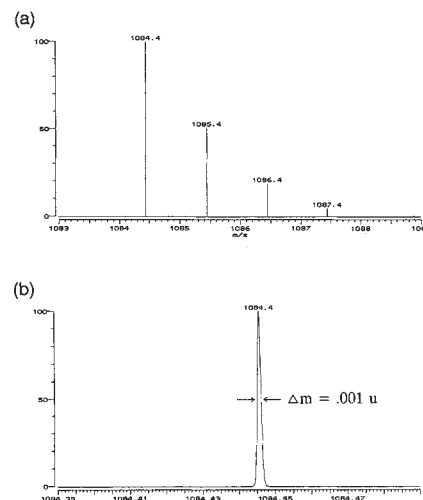


FIG. 2 High-resolution MALDI mass spectrum for [Arg⁸]-vasopressin: *a*, narrowband acquisition from m/z 1,080 to 1,090 showing the isotopic cluster peaks for the protonated molecular ion, and *b*, data plotted with an expanded mass axis to show a mass resolution of 1,100,000.

About 1 μ l of solution is delivered to a stainless steel sample probe and allowed to evaporate to dryness before insertion into the vacuum lock of the ion source. When the sample is irradiated with a laser pulse, extensive ionization occurs. The ions are rapidly extracted from the source region and injected into a long quadrupole ion guide. The purpose of the ion guide is to focus the ions and enable them to pass through the intense fringing fields of the superconducting magnet²⁸⁻³². As the ions exit from the quadrupole ion guide, they are focused by the magnetic field and trapped inside the FTMS analyser cell. After a delay time of 10 s, the ions are accelerated by a radiofrequency pulse and the image current signal produced by their coherent cyclotron motion is amplified and digitized. A minicomputer with a floating-point array processor calculates the Fourier transform of the time-domain image current signal and generates a spectrum showing the cyclotron frequencies of all the trapped ions.

High mass resolution

Several peptides and small proteins have been analysed successfully by the prototype MALDI-FTMS instrument. Figure 2*a* shows

a high resolution mass spectrum for $[\text{Arg}^{\text{K}}]\text{-vasopressin}$. One laser pulse produces a signal-to-noise ratio greater than 2,500:1. The base peak at m/z 1,084.446 is the 'monoisotopic' peak for the intact protonated peptide. It is isotopically pure; that is, all the carbon atoms in the molecule are ^{12}C , all the nitrogen atoms are ^{14}N , all the oxygen atoms are ^{16}O , etcetera. The three peaks at higher mass, each separated by 1 dalton, result from incorporation of one or more of the less abundant isotopes into the molecule, with ^{13}C at a natural abundance of 1.11% being the main contributor. It is difficult to measure the mass resolution in Fig. 2a because the peaks are so sharp, but the expanded mass scale in Fig. 2b shows that the monoisotopic peak is only 0.001 daltons wide, which gives a mass resolution of $m/\Delta m = 1,084/0.001 = 1,100,000$. This is believed to be the highest mass resolution ever reported for MALDI-produced ions.

In FTMS there is a trade-off between mass range and mass resolution. In order to achieve high mass resolution, as in Fig. 2, the cyclotron resonance signals produced by the ions must be digitized for several seconds. To avoid overflowing the buffer memory in the data system, therefore, the signals are passed through a mixer circuit that down-shifts the frequencies to a range that is more easily digitized. This is called the 'narrowband' detection mode. In Fig. 2, for example, 524,288 data points were acquired over the mass range m/z 1,080 to 1,090 in order properly to represent the high resolution peaks.

Figure 3a shows a MALDI mass spectrum for bovine insulin B-chain. For this spectrum a very small amount of peptide,

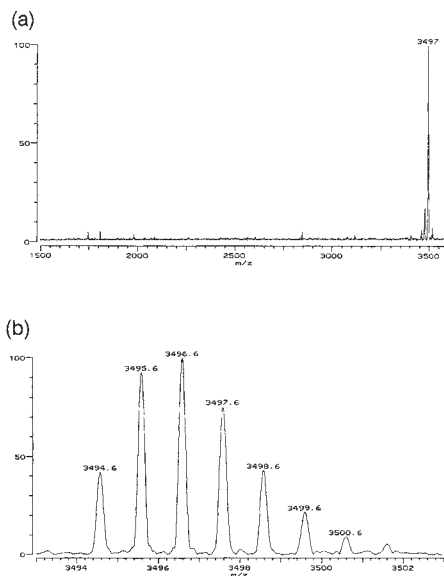


FIG. 3 MALDI-FTMS spectrum for 2 fmol of bovine insulin B-chain (broadband acquisition with 100 laser shots summed): a, protonated molecule is shown as the base peak in the mass spectrum, and b, expanded mass scale shows the resolved isotope peaks. The peak at m/z 3,500.6 corresponds to detection of 30 million molecules, or 50 amol.

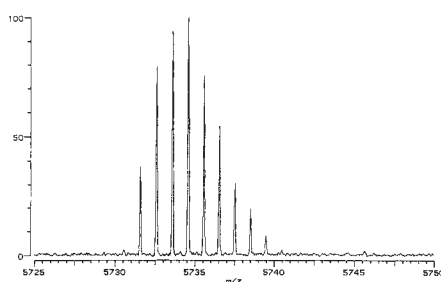


FIG. 4 High-resolution MALDI-FTMS mass spectrum for bovine insulin (narrowband acquisition with one laser pulse). The mass resolution is $m/\Delta m = 90,000$.

2 fmol, was pipetted onto the sample probe. Excellent signal-to-noise (80:1) is achieved after summing 100 laser shots, and the mass resolution is 20,000. In Fig 3b the same spectrum is expanded to show the numerous isotope peaks for the protonated molecule. This spectrum was acquired using the 'broadband' acquisition mode. It provides lower overall mass resolution, but a much wider mass range, m/z 1,500 to 3,600, is detected for each laser pulse.

The ultimate detection limit of MALDI-FTMS can be estimated by examining the dynamic range of the resolved isotope peaks. For example, there are eight distinguishable isotope peaks in Fig. 3b and the seventh one, labelled m/z 3,500.6, has a fractional abundance of 2.5%. This peak corresponds, therefore, to the detection of $2.5\% \times 2 \text{ fmol} = 0.05 \text{ fmol}$, or 30 million molecules, of the peptide. In other words, the analyte itself is actually a mixture with many isotopic components, and the trace component at a level of 2.5%, representing 30 million molecules, can be readily detected. This calculation illustrates dramatically the high ionization efficiency of MALDI and the potential of the method for future development.

Figure 4 shows a MALDI-FTMS mass spectrum of bovine insulin. The signal-to-noise ratio is about 50:1 for one laser pulse. The peaks of the isotope cluster for the intact protonated bovine insulin molecules are clearly seen at a mass resolution of 90,000. Several researchers have reported MALDI mass spectra of bovine insulin, obtained with both TOF³³ and quadrupole ion trap^{34,35} mass spectrometers. An advantage of the FTMS method is that higher mass resolution yields higher mass measurement accuracy and the capability of distinguishing impurities that are of similar mass²⁰.

Prospects

It is clear now that MALDI can be very effectively mated with an external ion source, quadrupole ion guide-type Fourier transform mass spectrometer to achieve very high mass resolution and femtomole sensitivity. One of the advantages of the external ion source method is that the conditions for efficient laser desorption can be optimized independently of the conditions for efficient, high-resolution ion detection in the

FTMS analyser cell. This is difficult to do in MALDI-TOF experiments because a change in the laser power or the source extraction potentials alters the flight time and the mass resolution.

The results reported here were obtained using a prototype FTMS instrument, and we are investigating several changes that might improve its mass resolution and detection sensitivity. The matrix materials and sample preparation procedures used thus far are about the same as are used for MALDI-TOF, but it is likely that different methods, not yet developed, will be more appropriate for the particular conditions of MALDI-FTMS. Future experiments will focus on extending these methods to molecules larger than bovine insulin and developing new methods for determining the structures of biomolecules. MALDI-FTMS may become a powerful method for studying the micro-heterogeneity and post-translational modifications of proteins. □

Robert T. McIver, Jr is a professor of chemistry and Yunzhi Li is a postdoctoral associate in the Department of Chemistry, University of California, Irvine, California 92717, USA. Their research is supported by a grant from the National Institutes of Health. Richard L. Hunter is president of IonSpec Corporation, 18009 Skypark Circle, Suite F, Irvine, California 92714, USA, and is the principal developer of the OMEGA 586 data system that is used with the Fourier transform mass spectrometer. For more information, fill in reader service number 100.

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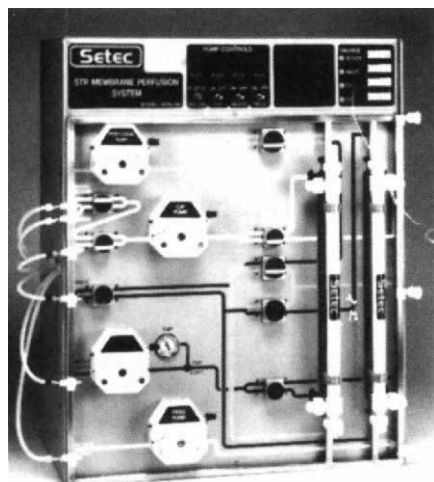
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Chemical concepts

In the biochemical arena — a new multi-purpose MALDI-TOF mass spectrometer, an automated ELISA/EIA processor, databases, a Windows-based chemical drawing package and a new micro-injection system.

A NEW tool for membrane research, phosphatidylinositol-specific phospholipase C (PI-PLC), can now be obtained from Kamiya Biomedical (*Reader Service No. 101*). Isolated from *Bacillus thuringiensis*, PI-PLC is a phospholipid degradation enzyme that cleaves phosphatidylinositol into diglyceride and myo-inositol-cyclic 1,2-phosphate. It is said not to act on phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol or sphingomyelin, and should be of use in research involving the presence, structure and biosynthesis of PI-anchoring proteins.

Setec has developed a new STR scalable membrane perfusion system that uses low speed peristaltic pumps to reduce cell shear damage (*Reader Service No. 102*). The



Setec's stirred tank reactor membrane perfusion system.

autoclavable system incorporates the company's 0.2 µm all-polypropylene Secor microfiltration modules, designed to offer low protein binding. It offers continuous feed/bleed and can later be upgraded from a manual system to an automated system.

■ Instrumentation

Hewlett-Packard has introduced its first matrix-assisted laser desorption/ionization time-of-flight mass spectrometer NATURE • VOL 370 • 4 AUGUST 1994



HP's first MALDI-TOF mass spec.

(MALDI-TOF) system, the HP G2025A (*Reader Service No. 103*). The instrument features a precision-machined probe for tightly controlled sample positioning and an ion optical design that maintains constant voltage, ensuring a stable acceleration potential that is insensitive to the concentration of desorbed ions. HP says that focusing repeller geometry enhances the sensitivity of the system by improving the efficiency of ion transmission from source to detector. The high-gain, dual microchannel plate detector is designed to limit charge depletion and potential signal loss, which can negatively impact sensitivity. A secondary ion generator increases conversion efficiency to increase signal gain for larger ions. Automation is facilitated by the sample preparation accessory for vacuum crystallization, designed to improve sample uniformity by producing small, homogeneous and evenly dispersed co-crystals. Control of the instrument, spectral acquisition, display and reporting are automated, and multiple samples can run unattended. Stated applications include determining the molecular weights of a wide range of samples and biomolecules (typically, femtomole to picomoles of sample are required and the mass range of the instrument is said to extend beyond 300,000 daltons). It can also be used to perform quality control on synthetic peptides and to confirm modifications; to screen samples prior to sequencing or further manipulation; to optimize enzymatic and chemical digest conditions; to corroborate peptide and oligonucleotide sequences; to characterize

glycosylation sites; and to help identify known and novel proteins. Prices start at under US\$125,000.

SF-41 Canterbury is Hi-Tech Scientific's dedicated stopped-flow system for the measurement of fast reactions at low temperatures (*Reader Service No. 104*). These include studies of biological catalysts which play an important part in reactions involving metalloenzymes. The unit offers a temperature range of -100 °C to 60 °C, and features an optical platform that allows absorbance or fluorescence measurements to



Hi-Tech's cryo stopped-flow system.

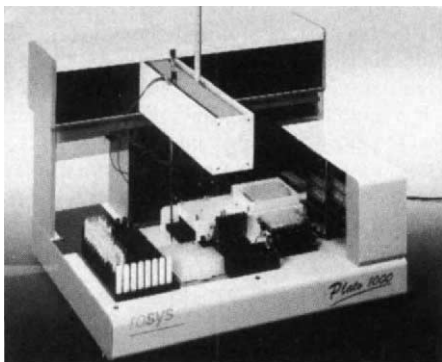
be made in the 200-1,000 nm wavelength range. The spectrometer is available with the MG-6000 rapid scanning diode array system. The flow lines and sample cell are housed within a thermostatically controlled environment; valves and syringes, however, are located outside this region to maintain optimum operation when the system is used at subzero temperatures. Temperature stability is said to be better than ± 0.1 °C over the temperature range. The instrument is intended for use with sample volumes as small as 75 µl. A pneumatic syringe drive is designed to ensure shot-to-shot reproducibility. Variable ratio mixing experiments can be performed, with ratios from 1:1 to 1:36 possible. A multi-mixing version is

PRODUCT REVIEW

also available. In addition, instruments can be tailored for applications that are oxygen sensitive using 'anaerobic' accessories.

■ Assays

The Plato 1000 from Rosys is a fully **automated, programmable ELISA/EIA processor** designed specifically for laboratories processing 35–100 samples per batch (*Reader Service No. 105*). Several different assays can be run on each batch of samples individually or according to a sample list. There is no need for user intervention between the loading of samples and the retrieval of the results. The system includes optional positive sample and plate identification, integrated sample handling (washable and disposable tips) for pre-dilution, dilution, sample preparation, controls and standards; fast reagent addition; plate incubation; washing; reading; and data analysis



Plato 1000 automated ELISA/EIA system.

(with customized presentation). An *x, y, z* robotic tool affects transport of the different plates between the processing modules, permitting random access of the plates so parallel processing of different tests can be automated. Up to eight plates can be processed in a single batch, with up to four in a temperature-controlled incubator tower at any one time. For higher throughput applications, Rosys offers Plato 1000's larger relative, the Plato 3300.

With the emphasis on flexibility, Anthos Labtec has introduced the **awl programmable microplate strip washer** (*Reader Service No. 106*). The washing procedure can be configured to the requirements of a particular test. All washing parameters, for example, type of plate, wash volume, plate agitation, pump pressure and soak times can be defined independently.

■ Databases/software

'Protecting Groups' is a chemical reaction **database of protection and deprotection chemistry** available from Synopsys (*Reader Service No. 107*). The database, which includes methods for protecting and deprotecting specific functional groups, contains over 20,000 reactions abstracted from more than 6,000 primary literature references. Updates will be provided featuring an esti-

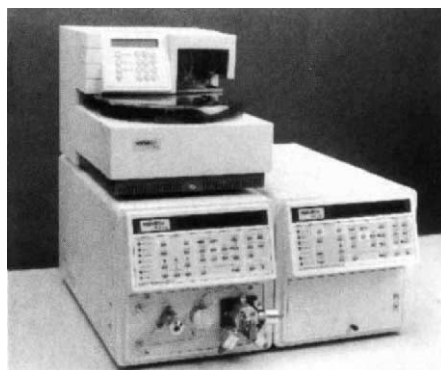
mated 1,000 new reactions per year. The database covers all the main areas of organic chemistry and includes information on regio- and chemoselective control. It incorporates cross-referenced data on stability and lability of the protected groups, and is supplied for use with the REACCS, ORAC and ISIS chemical database systems.

Version 2.0 of TAPP, a personal computer **database of thermochemical and physical properties** from E S Microwave, is now shipping (*Reader Service No. 108*). The database contains properties of over 17,000 solid, liquid and gas compounds. Included among these are details of crystal structure, density, thermal expansion, elasticity, thermal conductivity, diffusivity, electrical resistivity, viscosity, surface energy, vapour pressure, specific heat, entropy, enthalpy and free energy. Both organic and inorganic systems are included. Data sources include the JANAF tables, compilations by NIST, IUPAC and other sources of property data. Users can add their own compounds and data or supplement the data supplied with TAPP. Property values can be exported as tab-delimited files to be read by your spreadsheet, word processor or plotting application. The equations and coefficients used to calculate these temperature-dependent properties are displayed at the click of a button and can also be exported. The list price for TAPP is US\$495. Free data updates will be provided.

Cambridge Scientific Computing announces the availability of CS ChemDraw for Windows, a **chemical drawing program for Windows** environments (*Reader Service No. 109*). The program enables users to incorporate chemical drawings and structures into Windows-based applications such as word processing programs, spreadsheets and graphics software. Two versions are available: US\$495 CS ChemDraw and \$795 CS ChemDraw Pro. Both include specialized tools for drawing chemical structures as well as general purpose drawing and text tools to illustrate structures and mechanisms. ChemDraw Pro, however, also includes user-definable templates, colour and tools for integrating CS ChemDraw structures with databases including Chemical Abstracts and MACCS. Graphics developed in CS ChemDraw can be incorporated in and edited using Microsoft Word for Windows or WordPerfect for Windows for inclusion in reports or presentations. CS ChemDraw and CS ChemDraw Pro for Windows run under Windows 3.1 and Windows NT on IBM/compatible PCs, DEC and Alpha systems.

■ Chromatography

For users of high-performance liquid chromatography (HPLC), Varian now offers a new **96-sample capacity autosampler** (*Reader Service No. 110*). The Star 9300 HPLC autosampler is designed to improve productivity in a laboratory by automati-



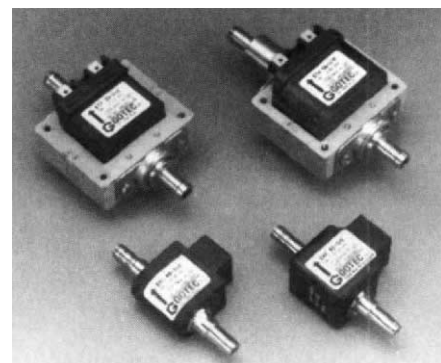
Varian's Star 9300 autosampler.

cally calculating run times. In addition, four removable racks allow sample preparation during operation of the autosampler. A specially designed valve system is said to cut down on the volume of sample needed to achieve precise loop-valve injections, and separate wash vials minimize carryover. A 5-ml vial that allows injections of larger sample volumes is an optional extra.

Gilson's new **832 rack temperature regulator**, based on the Peltier thermoelectric effect cells, provides heating and cooling of samples in thermostatically controlled racks and is designed to offer several advantages over standard liquid-circulating cryothermostats (*Reader Service No. 111*). It is intended for applications that require controlled sample heating and cooling, for example, to conserve the integrity of biological samples, to prevent evaporation of volatile solvents and to speed up enzymic reactions or chemical derivatizations. Designed for use with Gilson's liquid handling devices, fraction collectors and sample preparation systems, the 832 rack temperature regulator provides independent heating or cooling of two cuvettes. The temperature range is 4 °C to 40 °C. Various rack types are available. Control of the unit is provided by integral menu-driven software using the built-in keypad, or by PC-based Gilson system controller software.

■ Gadgets

E Clark & Associates announces the new Gotec line of **miniature oscillating piston pumps** intended for liquid handling applications including solvents, water, saline solu-



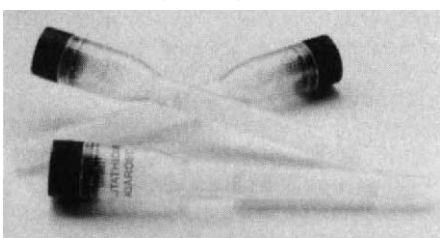
Pumps in miniature — see the Gotec line.

tion and other liquids (*Reader Service No. 112*). Gotec pumps, which feature a design that automatically limits pressure and eliminates potential overloading, are self-priming to 5 or 10 feet, depending on the internal check-valve configuration specified. The design also eliminates the need for shaft seals. Various models are available, with capacities ranging from 10 to 120 litres per hour and from 0 to 10 bar (0 to 140 p.s.i.). The pumps can be configured with brass, stainless steel or plastic end fittings.

With the new high-resolution **miniature colour camera** available from FORT, all of the camera controls and connections, including mains power, are accessible from the front panel (*Reader Service No. 113*). This feature is designed to eliminate time-wasting fumbling at the back of the camera during set up. The camera used is the Toshiba IK-48PK, one of a new generation of high-resolution 0.5-inch CCD cameras. In the FORT housing, it is known as the FTA-IK-48PK. The camera features an automatic electronic shutter with exposure times from 1/50th to 1/50,000th s, permitting hands-free operation and automatic white balance. It is available as standard for mains-powered operation on any supply in the range 100–240 VAC, 50/60 Hz without adjustment. Alternatively, it can be supplied for either mains or battery power. The unit provides either composite video and Y/C or S-video (S-VHS) with RGB as an option. It is said to be compatible with all picture monitors, video printers and recorders working to the PAL System 1 standard.

■ Extraction/purification

Sigma Chemical's **prepacked glutathione-agarose columns** are intended for fast and convenient protein purification (*Reader*



Sigma's prepacked purification columns.

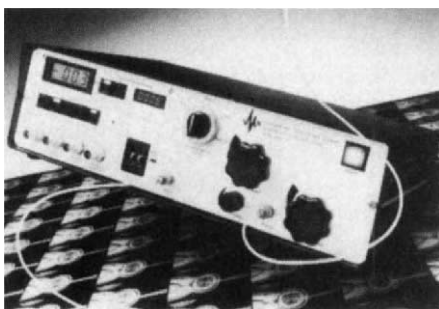
Service No. 114). Due to the affinity of glutathione-S-transferase to glutathione-agarose, the company says that cloned proteins fused to glutathione-S-transferase can be purified without denaturation. Each column contains 2.5 ml of glutathione-agarose with a stated binding capacity of 10–20 $\mu\text{mol ml}^{-1}$.

Perform solids extraction, evaporation and solvent recovery in the same unit with the new **highspeed analytical extractor** from Organomation Associates (*Reader Service No. 115*). The Sox-Evap extractor for solids features a glassware configuration that is said to provide up to 98 per cent solvent

recovery. The system incorporates a combination Soxhlet extractor and a KD flask for nondryness evaporation. It is supplied with either a 700 W water bath to accommodate 125- and 250-ml flasks, or a 1,100 W bath to handle 500-ml flasks. The eight Soxhlet condensers are connected to a central solvent collection vessel mounted on the centre tube which is easily drained. Prices range from US\$3,900 to \$6,200.

■ Equipment

PLI-100 is the new **pneumatic 'pico' injector** from Medical Systems (*Reader Service No. 116*). Three modes of injection are provided: internally timed, externally triggered and gated. The problem of pipette clogging has been overcome by a regulated balance



Pico injector system.

pressure. The need for backfilling the pipette can be eliminated due to a built-in vacuum generator for filling the pipette from the pipette tip. There is also a holding vacuum for suspended cells (for example, mouse oocytes). Injection and vacuum functions can be controlled by foot controls, leaving hands free for micromanipulations.

New from Fisher Scientific, an **Accumet titrator system** that is four precision meters in one (*Reader Service No. 117*). The system consists of the Accumet 150 titration controller with the Accumet coulometric KF titrator module (each sold separately) to perform titrations as well as four other measurements: conductivity, ISE, pH and mV. In addition, up to four titrator modules can be 'daisy-chained' to allow multiple titrations to be performed simultaneously. For laboratories that do not require the additional measurement modes, the KF titrator module can be paired with the Accumet 100 Controller to provide a 'titration only' unit. As with the Accumet 150, multiple titrations can be performed simultaneously by connecting additional titrator modules.

The new Model 501 **relative viscometer** from Viscotek is designed as an alternative to glass tube systems (*Reader Service No. 118*). The setup offers high throughput, on-line automation, fully enclosed operation for safety purposes and is intended to provide reduced solvent consumption over more traditional methods. The entry-level, semi-automated system can be upgraded to one that is fully automated for unattended opera-

tion. The system can also be coupled to a Zymark laboratory robot. The Model 501 can be used to determine relative viscosity, specific viscosity, inherent viscosity, reduced viscosity, intrinsic viscosity, absolute viscosity and molecular weight. Applications reports are available for analysing polyesters, mineral oils, polyolefins and polyamides. □

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